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Research Article

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Phenolic profile changes of grapevine leaves infected with *Erysiphe necator*

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Abstract

BACKGROUND: Powdery mildew in grapevine is caused by *Erysiphe necator* and its control requires many chemical treatments. Numerous efforts are being made to improve disease management to achieve crop sustainability goals. The exogenous induction of plant immune responses is one of the most encouraging strategies currently being developed. The objective of this research was to analyse differences in phenolic compound concentrations in *E. necator*-infected leaves of two varieties of *Vitis vinifera*, Tempranillo and Tempranillo Blanco, using ultra performance liquid chromatography coupled with mass spectrometry. To understand the susceptibility of the varieties, *in vitro* assays using whole leaves were done.

RESULTS: Differences in susceptibility between varieties were found in the early stage of the disease. In both varieties, total phenolic compounds were higher in infected leaves; however, hydroxycinnamic acid, anthocyanins and stilbenes were higher only in Tempranillo. Twenty-six compounds showed differential responses to the fungal disease in Tempranillo, but only two in Tempranillo Blanco: syringa resinol, which was not detected in diseased leaves; and gallocatechin, which increased at 5 days post inoculation. In Tempranillo, four anthocyanidins, six hydroxycinnamic acids, mainly feruloyl derivates, and epigallocatechin gallate were higher in infected leaves at the beginning of the infection, whereas (−)-epicatechin and protocatechuic hexoside contents were lower.

CONCLUSION: Disease-induced changes in phenolic compound biosynthesis were found. The increase in anthocyanidin content and flavan-3-ol galloylation could have a role in delaying *E. necator* growth in Tempranillo.
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Supporting information may be found in the online version of this article.

Keywords: *Vitis vinifera*; vine; powdery mildew; phenylpropanoids; plant defence

1 INTRODUCTION

Powdery mildew is one of the most important diseases of grapevine. It is caused by the fungus *Erysiphe necator* which is an obligate ectoparasite of species belonging to the *Vitis* genus, particularly *V. vinifera* varieties. Most *V. vinifera* varieties are highly susceptible; any green tissue of the vine can be parasitized by the fungus. Under favourable climatic conditions, namely moderate temperatures and high relative humidity without precipitation, the pathogen can generate numerous cycles of infection producing a large number of conidia, and thus causing substantial yield and economic losses.[1] Control of powdery mildew requires the intensive use of fungicides from flowering to veraison and even up to harvest time.

These practices, in addition to causing a negative ecological footprint, lead to the emergence of resistance to the most commonly used products; thus making them less and less effective,[2,3] while moving away from the global goal of sustainability in agricultural systems. One alternative for the sustainable management of plant diseases is based on the design of strategies that trigger a defence response in the plant to overcome fungal attack.[4] Because fungal diseases, especially powdery mildew, cause serious yield and quality losses and increase production costs all over the world, new resistant varieties are being developed in grape-breeding programmes.[5] In addition, different species and interspecific hybrids have been considered because of their resistance to powdery mildew.[6]

There are two steps to plant immunity. First, the pathogen has to overcome innate barriers, such as pathogen-associated molecular pattern-triggered immunity (PTI).[7] To overcome PTI, pathogens develop chemical signals, named effectors. The second step starts when the host cell recognizes the effectors with resistant proteins associated with gene-to-gene resistance. After recognition, effector-triggered immunity (ETI), a cascade of genes related to defence, is activated.[7] The mechanisms involved include deposits of callose at the infected points, and increases

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in peroxidase activity and phenolic compound synthesis that lead to cell wall strengthening and phytoalexin production. Both types of immunity seem to share downstream signalling machinery.[8] ETI processes are based on a co-evolutionary history, as seen in the relationship between American *Vitis* varieties and *E. necator*.[9] The existence of resistant genes in European *Vitis*, Kishmish Vatkana and Kara Djandal,[10] without a co-evolutionary history, suggests that other defence mechanisms recruit genes from the developmental signalling pathway and direct them towards the immunity pathway.[7]

Notable attention has recently been directed at secondary plant metabolites as stimulants of the plant immune system against pathogens.[4] In this respect, phenolic compounds have attracted interest because of their biological activity as antioxidants, particularly as natural fungicides. These compounds support plant resilience to stress caused by biotic and abiotic agents.[11] They play a central role in protection against damage due to ultraviolet (UV) light and in plant defences at distinct points. These compounds constitutively represent chemical barriers to infection that can increase after contact with a fungal pathogen. In addition, they can confer resistance through post-infection activation; acting as a defence response inducer, buffering the plant defence reactions or producing fungitoxic activity.[12–14] The pattern of gene expression shows large differences in tissue growth among cultivars, organs and varieties.[15] Accumulation of these compounds also depends on environmental conditions and cultural practices. All these factors interact, giving this pathway remarkable complexity and plasticity.[16,17] The biosynthesis of phenylpropanoids involves a cascade of oxygenases, oxidoreductases, ligases and transferase enzymes, which lead to the acylation, methylation, glycosylation and hydroxylation of phenols[11]; these compounds are expressed by structural genes controlled through *v-Myb* myeloblastosis viral oncogene homolog (MYB) transcription factors and MBW complex (ternary protein complex formed by MYB, bHLH (basic helix–loop–helix) and WD40 (repeat regulatory protein family)).[11,18]

In grapevine leaves, flavonols and phenolic acids are the main families that participate in constitutive plant defences,[19,20] and are the most abundant phenolic compounds in leaves. Stilbenes are the main phytoalexins of grapevine,[21,22] and are present in the lowest quantities, along with anthocyanidins.[15,23] Differential phenolic production between healthy and diseased plants has been associated with the resistance or tolerance of some cultivars to fungi, bacteria and other pathogens.[14,24] Fast pathogen recognition is a key factor in resistance. During the interaction between a plant and pathogen, the first stage of the defence mechanism involves the rapid accumulation of phenols at the infection site, the function of which is to stop or slow growth of the pathogen.[19,25]

Observations in the field and in preliminary laboratory trials showed greater powdery mildew susceptibility in Tempranillo Blanco than in Tempranillo (unpublished data), which coincides with comments by grape growers. Because these varieties are closely related genetically, the differential response to fungal attack could be due to a difference between the profiles of the phenolic compounds, especially anthocyanins. In this research, changes in phenolic compound metabolism in healthy and diseased leaves of Tempranillo and Tempranillo Blanco cultivars were analysed.

2 MATERIALS AND METHODS

2.1 Susceptibility assays

Plant material was obtained from cuttings of grapevine varieties cultivated in the experimental field of La Grajera (La Rioja, Spain; coordinates +42′ 26′ 37.52″, −2′ 30′ 56.30″ at an altitude of 384 m). Tissue samples were collected from Tempranillo (clone RJ43), Tempranillo Blanco, Mazuelo and Kishmish Vatkana, and the samples were used as positive and negative references in the susceptibility assays. Cuttings were sterilized at 50 °C for 30 min before propagation. To promote rooting, indole butyric acid was applied at 0.5 mg mL^{-1}. Plants were placed in a growth chamber with a 16:8 h light/dark photoperiod, temperature regimen of 24–16 °C at 60% relative humidity. Primary inoculum of *E. necator* conidia was obtained from infected leaves taken from La Grajera vineyards. The fungus was grown on detached leaves of var. Mazuelo that had been sterilized previously with 0.3% sodium hypochlorite solution for 30 s, rinsed four times with sterile distilled water and dried using sterile paper towels. Whole leaves were placed in separate Petri dishes (9 cm) containing 1% agar. Passes were made every 21 days for fungus maintenance. When plants reached the ten-leaf stage they were used in the experiments.

Four fully expanded leaves were collected from the second to fourth positions of the shoots for each variety. The leaves were subsequently sterilized and placed on agar plates as described above. The assay was repeated three times. Leaves were inoculated using a vacuum inoculation tower (50 cm diameter × 120 cm height) to obtain massive inoculation and uniform distribution of conidia[9]; settling of conidia for 10 min was carried out after stopping the vacuum pressure. Two researchers independently rated powdery mildew growth at 7 and 14 days post inoculation (dpi) using a binocular microscope (Leica EZ4 D, Leica Microsystems, Germany). Infection data were recorded using the scale: (1) no growth, (2) presence of hyphae, (3) conidiophore initiation (0–1 conidia), (4) >75 % developing conidiophores (2–6 conidia), and (5) >50% developed conidiophores and non-visible to the naked eye. In the scale, categories 6 to 8 all contained conidiophores that were completely developed, the differences in these categories were in the amount of the leaf surface occupied by fungus that was visible to naked eye: (6) <25%, (7) >25 to <75% and (8) >75%.

2.2 Chemicals and reagents

Methanol [liquid chromatography–mass spectrometry (LC–MS grade)], acetonitrile (LC–MS grade) and hydrochloric acid were from Scharlau (Barcelona, Spain). Myricetin, (+)-catechin, *trans*-resveratrol, malvidin, gallic, *p*-coumaric and formic acids (LC–MS grade) were from Sigma-Aldrich Chemical Co. (Steinheim, Germany).

2.3 Obtention of leaf extracts

Leaf extracts were taken from inoculated and healthy plants. The plants were obtained from cuttings of Tempranillo and Tempranillo Blanco varieties cultivated in the experimental field of La Grajera (La Rioja, Spain). Throughout the experiment, plants were maintained in a growth chamber under the conditions described above. Leaves from whole plants were cleaned with 0.3% sodium hypochlorite solution by spraying, rinsed with sterile distilled water and left to dry completely. Four plants of each variety were inoculated by spraying (sprayer from Preval, Fenioux, France) with a conidial suspension (>1×10^5 conidia mL^{-1}) in water with 0.001% Tween. For the controls, another four plants were sprayed with conidia-free solution. One leaf was removed from each plant at 1, 5 and 14 dpi and was immediately frozen at −80 °C until further analysis. For the extracts, leaves were ground to a powder with liquid nitrogen. One gram of ground leaf in

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10 mL of 0.1 M HCl 80% methanol solution was extracted with two consecutive 15-min cycles of sonication at 4 °C in total darkness.[24] Extracts were centrifuged for 30 min at 11000 g and 10 °C. The supernatant was filtered (0.45 µm PTFE filters) and kept at −20 °C until analysis.

2.4 Ultra performance liquid chromatography - diode array detector - mass spectrometry analysis of phenolic compounds

Leaf extracts were filtered (0.22 µm pore size) and injected through a Waters Acquity C_{18} column (1.7 µm, 2.1 mm × 100 mm; Waters, Milford, MA, USA), protected by a guard column with the same characteristics in an ultra performance liquid chromatography (UPLC) system. For hydroxybenzoic acids, flavonols, flavan-3-ols and stilbenes, the UPLC was coupled to a micrOTOF II high-resolution mass spectrometer equipped with an Apollo II electrospray/atmospheric pressure chemical ionization source in negative ionization mode, and a DAD detector at 280 and 360 nm (Bruker Daltonik, Karlsruhe, Germany). Separations were performed using acetonitrile/formic acid 0.1% and water/formic acid 0.1% (mobile phase A and B respectively) as solvents. Elution was carried out at a flow rate of 0.45 mL min^{-1} with an injection volume of 2.5 µL under the following conditions: 1% A for 1.5 min, a gradient from 1% to 8% A for 3.5 min, 8% to 12% A for 1 min, 12% A for 0.5 min, 12% to 14% A for 0.5 min, 14% A for 1 min, 14% to 22% A for 2 min, 22% to 30% A for 2.5 min, 30% to 90% A for 1.5 min and 90% for 1 min, in an oven at 40 °C. A 3-min equilibration period was allowed between injections.

For anthocyanidins, UPLC analyses were carried out on Shimadzu Nexera system (Kyoto, Japan), coupled to an AB Sciex 3200QTRAP® mass spectrometer (Sciex, Framingham, MA, USA), equipped with electrospray interface (ESI Turbo V™ source) operating in positive mode. Separations were performed using acetonitrile/formic acid 2% and water/formic acid 2% (mobile phase A and B respectively) as solvents, a flow rate of 0.45 mL min^{-1} and injection volume of 2.5 µL were used under the following conditions: 1% A for 1 min; 1% to 8% A for 1 min; 8% A for 3.2 min; 8% to 12% A for 1 min; 12% A for 0.5 min; 12% to 14% A for 0.5 min; 14% A for 1 min; 14% to 22% A for 2 min; 22% to 30% A for 3 min; 30% to 90% A for 1.5 min; 90% A for 0.5 min.

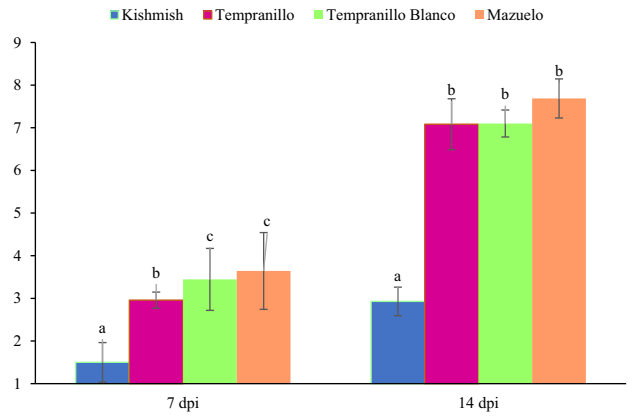

Figure 1. Development of *Erysiphe necator* on leaves of two Tempranillo varieties at 7 and 14 days after inoculation based on a scale from 1 (no infection) to 9 (>90% occupied by the fungus and visible to the naked eye). Mazuelo and Kishmish varieties were used as positive and negative controls, respectively. Different letters indicate significant differences.

Identification and quantification of compounds with negative ionization was performed using Bruker Compass DataAnalysis 4.2 software. Identifications were determined according to exact mass (monoisotopic) where the full range acquisition covered a mass spectra (*m/z*) range of 100–800. Anthocyanidins were analysed with Sciex Multiquant 3.02 software. Identifications were performed on the basis of the *m/z* of the ion mixtures generated by the breakdown of ionizable compounds in the sample. Quantitative determinations were carried out by using the following curve of standards: myricetin for flavonols, (+)-catechin for flavan-3-ols, *trans*-resveratrol for stilbenes, malvidin for anthocyanidins, gallic and *p*-coumaric acids for hydroxy benzoic and cinnamic acids. The data are presented as equivalents of the standard with similar chemical structure per gram of fresh leaf weight (µg Eq g FW^{-1}).

2.5 Statistical analysis

Data were analysed using SPSS Statistic v.23. To explore possible differences between varieties in their susceptibility to powdery mildew, a one-way analysis of variance (ANOVA) was used. The results are presented as mean ± SD with significant differences detected at the 0.05 level. Statistical significance in treatment (control/disease), colour (red/white) and day (1, 5 and 14 dpi), along with the interactions between them for total contents and individual compounds were assessed by multivariate analysis. Differences at the 0.05 level were considered. Principal component analysis (PCA) was used to examine the main compounds contributing to variability among samples. This analysis distributes the compounds in dimensional space based on their association with samples lying in the same direction.

3 RESULTS AND DISCUSSION

3.1 Susceptibility assays

Susceptibility to *E. necator* infection was evaluated using *in vitro* assays on whole leaves. Differences between varieties were found at 7 dpi: Tempranillo Blanco leaves presented conidiophores with several conidia, whereas in Tempranillo, the conidiophores were beginning to form the first conidia (scale: 3.4 and 2.9 respectively, $P < 0.05$). Schnee et al.[21] studied the growth cycle of *E. necator* in *in vitro* assays on leaf discs. They observed that the duration of the cycle depended on the resistance or susceptibility of the variety. In susceptible varieties, appressoria formation takes place between 24 and 48 h, whereas penetration peg and haustorium formation takes place between 3 and 7 dpi. Accumulation of phenolic compounds at the appressorium site, at the host cell wall and at neighbouring cell walls was reported in response to infection.[8,21]

After 6–7 dpi, further mycelial development occurred and sporulation density increased. In our research, at 14 dpi both varieties showed the same susceptibility and developed conidiophores with numerous conidia that were visible to the naked eye and covered almost the whole leaf surface (Fig. 1).

3.2 Phenolic compound profiles

3.2.1 Analysis of total contents of phenolic groups in response to infection

Six groups of phenolic compounds were analysed: anthocyanidins, flavan-3-ols, flavonols, stilbenes, and hydroxybenzoic and hydroxycinnamic acids. Hydroxycinnamic acids were the most predominant (80%–90%), as reported previously,[12,15,23] followed by flavonols (3.9%). In other research, flavonols were one of the most abundant phenols.[15,23] Because solar exposure induces accumulation of these compounds, the low content in this

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research may be due to the lack of exposure of the assay plants to solar radiation.[24,25] Anthocyanidins were the least abundant, but it should be noted that these compounds were analysed using different chromatographic methods.

To compare the total contents of each group between diseased and healthy leaves, ANOVA was undertaken. Total phenolic compound content increased significantly in both varieties (42% and 30% at 14 dpi, $P < 0.05$, in Tempranillo and Tempranillo Blanco respectively) (Fig. 2). Similar results were found in 23 *Vitis* varieties, including resistant and highly susceptible ones; in all cases, increases in total phenol content were found although there was variability among varieties.[26,27] An increase in antioxidant activity and phenolic compounds after powdery mildew infection has been described in 15 *Vitis* cultivars,[28] suggesting a relationship between the two.[29] This pathway starts with the phenyl alanine amino acid leading to *p*-cinnamic acid catalysed by phenyl alanine ammonia lyase whose activation is often observed in plant defence.[20,30] *p*-Cinnamic acid is converted to coumaric acid, which is divided at different branches. When coumaric acid is catalysed by coumarate-3-hydroxylase it becomes hydroxy phenolic acid, when catalysed by chalcone synthase it becomes flavonoid (flavonols, flavan-3-ols, flavanones and anthocyanidins), and when catalysed by stilbene synthase it becomes resveratrol and its derivatives.[11] In this research, concentrations of hydroxycinnamic acids and stilbenes at 14 dpi were higher in diseased than in healthy Tempranillo leaves (1.5 and 3.5 times, $P < 0.05$ respectively), whereas the flavonoid content did not change significantly. Stilbenes are usually found in low amounts, mainly induced by pathogens.[12,19,22] The low concentrations at which these compounds are found may be due to a dilution effect because they are expressed at the point of infection, without being transferred to adjacent cells.[21]

A compound rearrangement was found among flavonoids which led to an increase in anthocyanidins at 14 dpi in diseased Tempranillo leaves (3.7 times, $P < 0.05$). Anthocyanidin compounds are not usually considered in most grapevine leaf extracts studies, yet where they were, they are found in low amounts.[21,23,31] These compounds contribute to the pigmentation of plant organs and are involved in UV protection.[32,33]

3.3 Analysis of principal components

PCA was used to analyse the relationships of the samples on a score plot (Fig. 3(a)); separation of the samples could be distinguished by the PCA loading plot (Fig. 3(b)). Principal components (PC) 1 and PC2 accounted for 65% of the total variance. PCA revealed distinct general metabolite profiles between the two cultivars, which were explained by PC2 with 23% of the variance. Tempranillo Blanco was on the positive side and was characterized mainly by hydroxyphenolic acids and flavan-3-ol compounds. PC1 separated Tempranillo diseased leaves at 14 dpi from the rest, and this sector was characterized by anthocyanidin and flavonol compounds.

3.4 Phenolic compound contents in response to infection

The chromatographic profiles of hydroalcoholic extracts from healthy and diseased leaves were qualitatively similar, except for

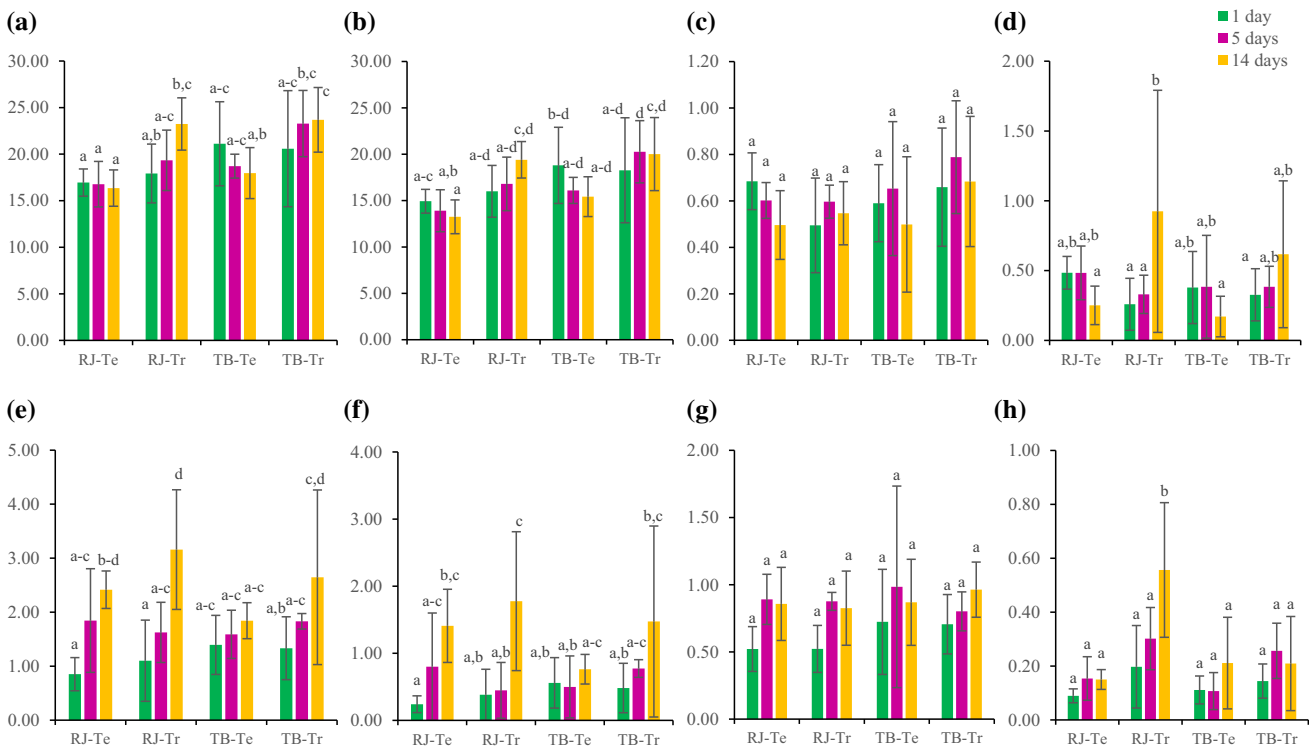

Figure 2. Total content of phenolic compounds (a), hydroxycinnamic acids (b), hydroxybenzoic acids (c), stilbenes (d), flavonoids (e), flavonols (f), flavan-3-ols (g) and anthocyanidins (h) of healthy and powdery mildew-infected grape leaf extracts. The extracts were analysed with two chromatographic methods: ultra performance liquid chromatography (UPLC) coupled to an AB Sciex 3200QTRAP mass spectrometer with an ESI Turbo V source in the positive mode for anthocyanins; and for the rest, UPLC coupled to a micrOTOF II high-resolution mass spectrometer with an Apollo II electrospray/atmospheric pressure chemical ionization source in negative ionization mode. RJ/TB, Tempranillo/Tempranillo Blanco; Te/Tr, healthy/diseased leaves; 1/5/14, days after inoculation. Data are expressed as mean (μg Eq g FW^{-1}) ± SD ($n = 4$). Different letters represent significant differences following Duncan's test ($P < 0.05$).

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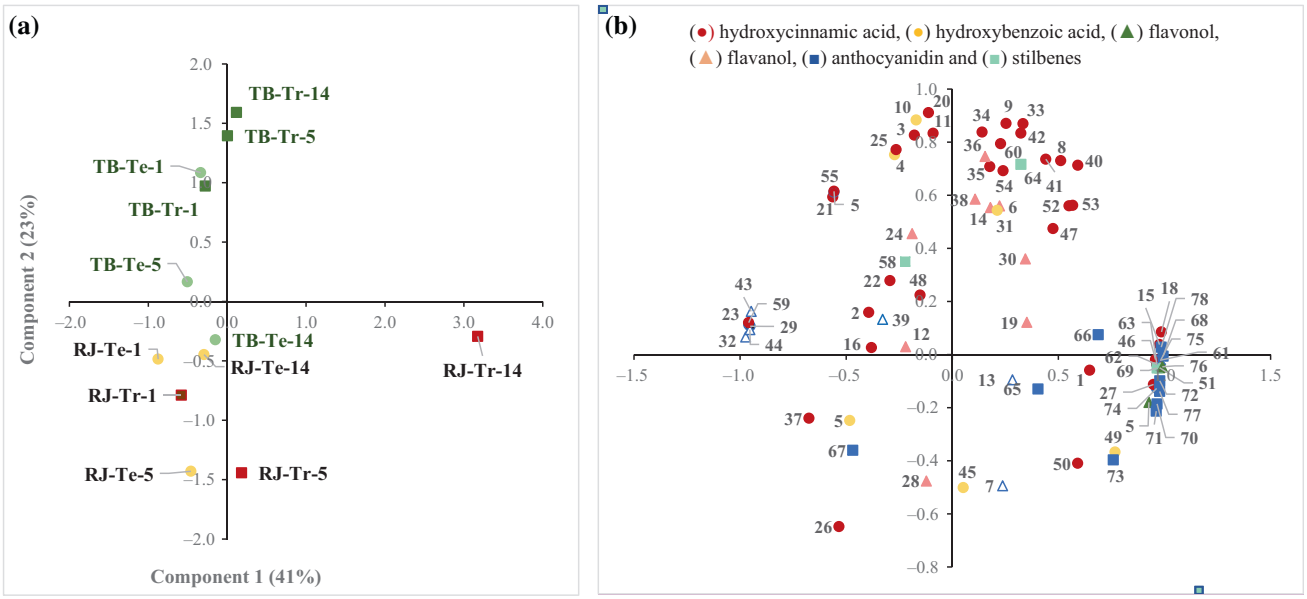

Figure 3. Principal component analysis of healthy and powdery mildew-infected grape leaf extracts (a) and score plot of extract data (b). Loading plot showing distribution of variables. RJ/TB, Tempranillo/Tempranillo Blanco; Te/Tr, healthy/diseased leaves; 1/5/14, days after inoculation. The equivalence between the number and the compound is shown in Supporting information Table S1. These results were obtained from extracts analysed with two chromatographic methods: ultra performance liquid chromatography (UPLC) coupled to an AB Sciex 3200QTRAP mass spectrometer with ESI Turbo V source in positive mode for anthocyanins; and for the rest, UPLC coupled to a micrOTOF II high-resolution mass spectrometer with an Apollo II electrospray/atmospheric pressure chemical ionization source in negative ionization mode.

syringa resinol (isomer 1) and caffeoyl hexoside (isomer 1). The chromatograms rendered 76 compounds, 70 of which were identified (Supporting Information Tables S1 and S2). Hydroxycinnamic acids were the most predominant with 35 compounds; coumaric (isomer 1) (4.4–5.8 μg g FW^{-1}) followed by caffeic (isomer 2), caffeoyl tartaric and feruloyl tartaric (isomer 3) acids (>0.10 μg g FW^{-1}) were the most abundant, as found by other authors.[15,16] They may be related to cell wall elongation and the formation of polymeric waxes on plant surfaces,[20] activities that take place more often in younger leaves than in old ones. Next were the anthocyanidins with 14 compounds; coumaroyl glucosides of peonidin (0.210–0.023 μg g FW^{-1}) and malvidin (0.097–0.016 μg g FW^{-1}) were the most abundant. Ten flavan-3-ols were identified and six of them were monomeric; (+)-catechin and (−)-epicatechin were the most abundant (0.026–0.100 and 0.306–0.096 μg g FW^{-1} respectively). Hydroxybenzoic acids, flavonols and stilbenes were the least diverse (five, three and three compounds, respectively).

Among all the compounds, syringa resinol and caffeoyl hexoside were the most notable (Supporting Information Figs S1 and S2). Syringa resinol (isomer 1) was not detected in diseased leaves of Tempranillo Blanco or in Tempranillo at 14 dpi. This compound has an important role in the formation of cell barriers,[11] and its loss could make it difficult to repair damage caused by the pathogen and so favour disease progression. Thus, its role in plant defence was not associated with host–pathogen recognition but rather to repair cell damage.[34] Caffeoyl hexoside was hardly registered in control leaves of Tempranillo (<0.001 μg g FW^{-1} at 1 and 5 dpi), being induced *de novo* in infected leaves (0.015 μg g FW^{-1}, $P < 0.05$); no difference was found in Tempranillo Blanco (mean value 0.066 μg g FW^{-1}). Different roles related to the defence response of caffeoyl hexoside have been reported; for example, phytoanticipins are present before infection, or have fungitoxic activity.[35] Caffeoyl hexoside may also be involved in structural defences. In the powdery mildew-resistant Regent variety, the caffeoyl hexoside content is higher than in non-resistant varieties.[30,36]

When healthy leaves of both varieties were compared, only nine compounds were present in distinct concentrations; eight were hydroxycinnamic acids, of which three were isomers of coumaric acid and three were tartaric derivates. However, when the disease response of each variety was compared, Tempranillo showed greater differences in disease progression than Tempranillo Blanco. Significant differences were found for 26 compounds in Tempranillo compared with 2 in Tempranillo Blanco. The genetic background of both varieties is similar because Tempranillo Blanco is the result of a natural mutation in Tempranillo involving complex rearrangements of the genome. This rearrangement was due to massive fragmentation of some chromosomes, and could have led to the loss of some genetic information,[37] which may include *Myb* genes involved in phenolic biosynthesis regulation.[18] This result suggested a specific defence response in this variety, as found by other authors.[17,30]

In Tempranillo, 14 hydroxycinnamic acids, 7 anthocyanidins, 3 flavan-3-ols, 1 stilbene and 1 hydroxybenzoic acid were found in different concentrations between control and infected leaves. At 1 dpi, coumaric acid (isomer 1), feruloyl tartaric acid (isomer 2) and the anthocyanidins, peonidin hexoside and coumaric derivates of peonidin and cyanidin, were more abundant. On the other hand, the level of protocatechuic hexoside was lower (approximately three times lower, $P < 0.05$). Protocatechuic hexoside has been associated with a reduction in respiration rate at high concentrations.[38] At 5 dpi, malvidin glucoside, ferulic acid, feruloyl malate and epigallocatechin gallate were accumulated, whereas the (−)-epicatechin level was lower in diseased leaves compared with healthy ones. Ferulic derivatives have been associated with resistance/tolerance to diseases, such as powdery[27] and downy[36] mildews and Botrytis.[30] In grapevine trunk disease

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caused by *Neofusicoccum parvum*, ferulic acid activates the secretion of a fungal phytotoxin involved in plant defence, which leads to programmed cell death.[39] Taken together, these results suggest that ferulic derivates could play an important role in the host–pathogen interaction.

Regarding flavan-3-ols, Atak et al.[40] analysed the content of some phenolic compounds in 22 *V. vinifera* and interspecific hybrid cultivars with different susceptibility to *E. necator* and reported increases in (+)-catechin, (−)-epicatechin and gallic acid after powdery mildew infection. However, these changes appeared to be independent of cultivar tolerance/resistance. For the anthocyanidins, cyanidin and malvidin glucosides are the most cited in relation to diseases, and their concentrations increase as the severity of powdery mildew increases in Cabernet Sauvignon and Sauvignon Blanc.[27] Similar results have been reported in Sangiovese plants infected by Bois Noir.[14] Cyanidin can even be induced *de novo* in plants infected by leaf roll virus, in the Merlot variety.[33]

The concentrations of the remaining significant compounds changed mainly at 14 dpi; among them, resveratrol hexoside, which increased in diseased leaves (4.99 times, $P < 0.05$), was the only difference found among the stilbenes. The fungal toxicity of these compounds is closely related to their chemical structure, with δ-viniferin and pterostilbene being the most active against pathogen mobility and disease development.[21,22] Therefore, viniferins have been proposed as markers to assess defence potential in grapevine disease resistance breeding programmes.[41] By contrast, resveratrol mainly accumulated in its glycosylated form in susceptible varieties.[14,42] This accumulation could be due to the defence response, but the earliness of pathogen recognition, leading to resveratrol synthesis, and the velocity of its oxidation is key to achieving resistance to pathogens.[21]

In Tempranillo Blanco, syringa resinol and gallocatechin at 5 dpi, were the only compounds showing significant differences ($P < 0.05$). For feruloyl tartaric acid (isomer 2) ($P = 0.09$), (−)-epicatechin ($P = 0.093$ at 5 dpi) and resveratrol glucoside ($P = 0.08$ at 14 dpi) the same trend as in Tempranillo was found, yet with distinct timing (Supporting Information, Fig. S2). It is possible that the low number of replicates used in this work may affect the results, which could suggest that (−)-epicatechin could be deviated to produce galloylated forms, and the esterified galloyl forms could play a role in delaying fungal growth.

When concentrations were compared between treated leaves, 13 compounds showed significant differences. Seven of them coincided with those mentioned above. For the other six, higher contents of the hydroxyphenolic acids, vanillin and galloyl hexosides, acetyl coumaric acid and feruloyl hexoside were found in Tempranillo Blanco, whereas ellagic acid and epigallocatechin concentrations were higher in Tempranillo. No differences were found among flavonols even though they have been associated with the defence response in grapevine; quercetin derivates are most cited in the literature.[14,25] Therefore, it is possible that these compounds could play a role in the defence of Tempranillo in the field, which would explain the greater differences in disease severity observed in the field (unpublished data) compared with *in vitro* assays.

4 CONCLUSIONS

In this research, the two varieties studied, Tempranillo and Tempranillo Blanco, presented distinct phenolic compound content profiles in response to infection by the pathogen *E. necator*. The results corroborate the wide variability in the regulation of phenolic biosynthesis among pathways based on variety. In the interaction between Tempranillo leaves and the pathogen, increases in anthocyanidins, galloylation of flavan-3-ols and accumulation of hydroxycinnamic acids were observed. However, these changes were not enough to prevent pathogen development. The delay in disease development in Tempranillo compared with Tempranillo Blanco might be related to the induction of caffeoyl hexoside, the accumulation of syringa resinol and the increase in feruloyl derivates and epigallocatechin gallate. These factors might play a role in the repair of structures damaged by the fungus and could also participate actively through fungitoxic or antioxidant activities.

Further evaluation of the phenolic content of resistant varieties is required to decipher the role of these compounds in plant–pathogen interactions. Activation of the immune response of traditional susceptible varieties through elicitors and the identification of specific natural bioactive compounds could constitute two new strategies that would be particularly useful in protecting vineyards against fungal pathogens.

AUTHOR CONTRIBUTIONS

Investigation, conceptualization, visualisation, methodology, writing—original draft, editing, supervision, funding: María del Mar Hernández. *Formal analysis, validation*: Carolina Castillo Río. *Formal analysis, validation*: Sara I Blanco González. *Conceptualization, writing—review, supervision, funding acquisition*: Cristina M Menéndez.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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