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The interplay between hormone signaling and defense gene expression in grapevine genotypes carrying genetic resistance against *Plasmopara viticola*

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Summary

The present study aimed to investigate plant defense related pathways during *Plasmopara viticola* infection in *Vitis vinifera* varieties. Plant material consisted of 'Chardonnay' (no *Rpv*), 'Regent' (*Rpv3-1*), 'Bronner' (*Rpv3-3+Rpv10*), 'Calardis Blanc' (*Rpv3-1+Rpv3-2*), and the breeding selection GF15 (*Rpv1+Rpv3-1*). Gene expression analysis was carried out for the varieties 'Regent', GF15, 'Bronner', and 'Chardonnay'. Hormonal quantification was performed for jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), indole-3-acetic acid (IAA), and trans-zeatin-ribose (tZR). The samples were collected from plants cultivated *in vitro* inoculated with *Plasmopara viticola* sporangia, and collected at 0, 1-, 3-, 5-, and 7-days post inoculation (DPI) for gene expression; and 0, 3, 5, and 7 DPI for hormonal quantification. The results showed an interaction between genotype and time post inoculation in gene expression and hormonal pathways linked with pathogen recognition. Both jasmonate and salicylic acids were involved in the resistance response. The role of stilbenes acting against the pathogen at different times was also confirmed. Changes in the expression of genes linked to cell defense were observed in all evaluated genotypes; however, genotypes with R-loci responded more quickly than the variety without R-loci, activating mechanisms of cell death, resulting in symptoms of hypersensitivity.

K e y w o r d s : *Vitis vinifera*; PIWI cultivars; vitiviniculture; disease resistance; plant breeding; gene expression.

Introduction

Grapevine downy mildew is caused by the biotrophic oomycete *Plasmopara viticola* (Berk. and Curt) Berl. & de Toni. This is one of the most challenging diseases in viticulture worldwide since it can negatively affect grape quality and yield. Currently, fungicide treatments are used to control downy mildew, mainly those based on the active ingredients metalaxyl + mancozeb (de Souza *et al.* 2018) and copper (Cabús *et al.* 2017); the latter being the only one accepted by the European organic farming regulation EC 889/2008. Other active ingredients are also used against downy mildew in viticulture worldwide, such as propineb, dithianon, fenamidone, mancozeb, folpet, cymoxanil + famoxadone, cymoxanil + maneb, iprovalicarb + propineb, benalaxyl + mancozeb, azoxystrobin, fosetyl-Al, and captan. However, phytosanitary treatments increase production costs and may result in negative impacts on the environment and human health (Gessler *et al.* 2011, Taylor and Cook 2018).

P. viticola originated in North America and since the second half of the 19th century, the species was disseminated throughout the world, causing devastation of European viticulture (Gessler *et al.* 2011). With rare exceptions, the cultivated grapevine varieties of *V. vinifera* species, which originated in Europe, are highly susceptible to this pathogen (Sargolzaei *et al.* 2020). Varieties with one or more *R-loci* are named as PIWI, from the German word "pilzwiderstandsfähig" that means grapevine disease resistance. *R-loci* conferring resistance to *P. viticola* (*Rpv* – Resistance to *P. viticola*), inherited from American and Asian *Vitis* species, were identified by quantitative trait loci (QTL) analysis (Maul *et al.* 2020).

Rpv1, located on chromosome 12, was inherited from the American species *Muscadinia rotundifolia* (2n=40) (Merdinoglu *et al.* 2003). *Rpv3* is located on chromosome 18 (Fischer *et al.* 2004). Up to now three different haplotypes at the *Rpv3* locus were characterized. *Rpv3-1* is the most common, and the first *Rpv3* haplotype described, inherited from *V. rupestris* (2n=38) and mapped from 'Regent' and 'Bianca' (Welter *et al.* 2007, Bellin *et al.* 2009). The *Rpv3-2* has been inherited from *V. rupestris* or *V. lincecumii* (Zyprian *et al.* 2016), and *Rpv3-3* inherited from *V. labrusca* or *V. riparia* (Vezzulli *et al.* 2019). The *Rpv10* locus is located on chromosome 9 and was inherited from Asian species *V. amurensis* (Schwander *et al.* 2012). All *Rpv* loci individually confer partial resistance to *P. viticola*, varying in intensity (Possamai *et al.* 2020). When two or more *Rpv* loci are combined in the same plant, they commonly result in additive interactions, increasing the level of resistance (Schwander *et al.* 2012, Venuti *et al.* 2013, Saifert *et al.* 2018, Zini *et al.* 2019). Additive ef-

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fects of *R*-loci are reported for combinations *Rpv1+Rpv3*, *Rpv3+Rpv10,* and *Rpv3+Rpv12*, based on increasing the resistance in bioassays (Schwander *et al.* 2012, Venuti *et al.* 2013, Saifert *et al.* 2018), or in field conditions (Zanghelini *et al.* 2019).

The most important *Rpv*s genes are placed in genomic regions rich in the nucleotide-binding domains leucine-rich repeats (NB-LRR) gene class (Merdinoglu *et al.* 2003, Welter *et al.* 2007, Schwander *et al.* 2012, Feechan *et al.* 2013, Venuti *et al.* 2013, Sapkota *et al.* 2019). However, until now, only *MrRPV1* was functionally characterized as an NB-LRR gene (Feechan *et al.* 2013). NB-LRR proteins are codified by *R*-genes and directly or indirectly recognize pathogen effectors (*R-Avr*), resulting in interplay with phytohormones, especially salicylic acid (SA) and jasmonic acid (JA), activating the defense pathways (Lolle *et al.* 2020). This resistance mechanism is known as effector-triggered immunity (ETI) (Jones and Dangl 2006).

Isolates of *P. viticola avrRpv3*- overcome the resistance conferred by *Rpv3* (Peressotti *et al.* 2010); however, when the *Rpv3* is combined with *Rpv12*, *avrRpv3*- pathogen effectors are recognized, and the defense mechanism is activated, restricting pathogen development (Venuti *et al.* 2013). However, non-additive effects were also reported for some genotypes containing pyramided *Rpv* loci (Saifert *et al.* 2018, Zini *et al.* 2019), suggesting the role of minor QTLs in determining the resistance phenotype. Plant reaction against the pathogen may change according to the tissue phenological age, and mechanisms of action depend on host resistance to the disease

Successful pathogen recognition triggers the ETI by activation of signal transduction pathways involving mitogen activated protein kinases (MAPK) and WRKY transcription factors, which in turn trigger primary immune responses (Thomma *et al.* 2011, Feechan *et al.* 2013). Examples of the immune response are accumulation of pathogenesis related (PR) proteins, reactive oxygen species (ROS), and phytoalexins; and in grapevines, particularly the activation of stilbene synthase (STS), resveratrol O-methyltransferase (ROMT) and glycosyltransferase (GT) enzymes (Sarris *et al.* 2015, Fröbel *et al.* 2019, Horsefield *et al.* 2019). The functioning of this system may result in a hypersensitive response (HR) that prevents pathogen development (Sarris *et al.* 2015, Devendrakumar *et al.* 2018). However, recently ETI was reported in partial resistant Georgian *V. vinifera* access without HR response (Sargolzaei *et al.* 2020).

The SA pathway is a plant cell defense inductor against biotrophic pathogens, such as *P. viticola*. However, challenging grapevine cells with mildews may also activate the JA pathway (Polesani *et al.* 2010, Guerreiro *et al.* 2016). This activation is ascribed to the lipid oxidation on the attacked cells, thus launching jasmonate synthesis, which culminates in a hyper-sensibility response against biotrophic pathogens (Choudhury *et al.* 2017).

The interplay between SA and JA in defense response pathways is mainly regulated by *WRKY70*, which in turn is regulated independently by *Non-expressor of PR1* (*NPR1*) and *AtMYB44* (Dong 2004, Shim *et al.* 2013, Caarls *et al.* 2015). Commonly, the role of antagonist by these hormones is reported (Shim *et al.* 2013). However, during the ETI, JA can be activated by SA receptors, such as NPR3 and NPR4, resulting in a joint action by these hormones (Liu *et al.* 2016). JA acts in the activation of Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase encoded by CORONATINE INSENSITIVE 1 (COI1). SCF^{COI1} promotes ubiquitination of the Jasmonate ZIM-domain (JAZ) and carries it to be degraded on the 26S proteasome. This episode causes the release of the Novel Interactor of JAZ (NINJA) that acts as a transcriptional repressor of the Tup1-type co-repressor TOPLESS (TPL). After, the transcription factor MYC2 is activated, and the JA related gene expression begins (Pauwels *et al.* 2010). Based on this context, the objective of the present work was to characterize the interplay between salicylic and jasmonic acid pathways, and the kinetic of gene activation (specifically genes involved in the stilbene metabolism) during downy mildew infection in genotypes carrying *R-loci* for the resistance to downy mildew.

Material and Methods

Plant material: The genotypes challenged with *P. viticola* were the susceptible *V. vinifera* variety 'Chardonnay', the PIWI varieties 'Regent' (*Rpv3-1*), 'Calardis blanc' (*Rpv3-1+Rpv3-2*), and 'Bronner' (*Rpv3-3+Rpv10*), and the advanced breeding selection GF.2004.0043.015 (thereafter called GF15, *Rpv1+Rpv3-1*), kindly provided by the grapevine breeding program of the Julius Kühn-Institute – Germany. The genotypes were selected to represent different *Rpv* combinations. All genotypes were used to perform phytohormonal quantification, and except Calardis Blanc, to perform gene expression analysis.

In vitro plant cultivation: The experiment was carried out in *in vitro* conditions. All genotypes were introduced *in vitro* from nodal segments and maintained *in vitro* conditions, using DSD1 culture medium (Silva and Doazan 1995) added to sucrose (2 %). Two plantlets were maintained in each cultivation flask (300 mL), supplied with 50 mL of culture medium, in room temperature of 25 °C with 16 h light photoperiod.

Downy mildew propagation and inoculation: Young leaves from the susceptible 'Cabernet Sauvignon' exhibiting typical downy mildew symptoms were collected from an experimental vineyard from the EPAGRI (Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina), Videira, Santa Catarina, Brazil. Leaf discs containing the sporulating lesions were collected and stored at -20 °C until use. Seven days before setting up the experiment, the sporangia were activated and cultivated on leaf discs excised from young leaves from susceptible 'Chardonnay' under *in vitro* conditions. Previously, 'Chardonnay' leaves were disinfected by a solution of sodium hypochlorite (1 %) for 2 min, followed by a triple wash with sterile distilled water (SDW).

Five leaf discs of 1.6 cm in diameter were placed abaxial side up on each 15 cm diameter Petri plate containing SDW-soaked filter paper. The inoculum was defrosted in cold SDW and immediately sprayed on the leaf discs, using a 500 $\mu L \cdot plate^{-1}$. The Petri plates were sealed and stored in the dark at room temperature. Twenty-four hours after inoc-

ulation (HPI) droplets were removed from the leaf discs and the cultivation was maintained under the same temperature conditions with a 16 h light photoperiod for the next six days, when the sporangia were collected in sterile water. The sporangia suspension was then adjusted to the concentration of $5x10^4$ sporangia mL^{-1}. All the procedures were performed in the flow chamber using previously sterilized materials.

A single droplet of the sporangia suspension was applied to the abaxial side of all mature leaves of the *in vitro* plantlets of all five genotypes. Leaves of the control plants were inoculated only with SDW. After inoculation, the flasks were sealed and kept in the absence of light at 25 °C for 24 h and afterwards maintained at the same room temperature in a 16 h light photoperiod. The experiment was completely randomized, with three replications and five flasks per repetition, totalizing 10 plants per experimental unit.

Expression of resistance-related genes: A sample (200 mg) of fresh leaves was collected at five different times after inoculation (0, 1, 3, 5, and 7 DPI) from plants of each repetition. The collected leaves were immediately frozen in liquid nitrogen and remained at -80 °C until use. RNA extraction and purification were performed using the commercial kit SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's recommendations. The quality of the RNA was confirmed by electrophoresis on agarose gel [1.5 %], denatured with formamide and stained with Gelred [1X]. Quantification was obtained using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific) and the concentration of all samples was standardized to 10 $ng \cdot \mu L^{-1}$.

Reverse transcriptase quantitative PCR (RT-qPCR) reactions were carried out using GoTaq Master Mix RT-qPCR Systems (Promega, Madison, WI, USA), according to the manufacturer's recommendations, in a StepOnePlus thermocycler (Thermofisher, CA, USA). The expression of genes related to SA and JA pathways were quantified, together with the endogenous putative gene controls *Actina*, *EF1-α*, *GAPDH,* and *Ubiquitin* (Tab. S1). The primer efficiency was established using six points of a 1:10 dilution. All reactions were performed in technical duplicate. The expression data were normalized by the geometric mean estimated by the geNorm algorithm (Hellemans *et al.* 2007) of the best two endogenous controls selected according to the most stable expression in a pilot experiment. The relative quantification method (Pfaffl 2001) was employed:

$$ratio = \frac{E_{target}^{\Delta CP_{target}^{(control-sample)}}}{E_{reference}^{\Delta CP_{reference}^{(control-sample)}}}$$

Hormonal analysis: A sample (500 mg) of fresh leaves from *in vitro* plants was collected at four different times after inoculation (0, 3, 5, and 7 DPI), immediately frozen in liquid nitrogen, and maintained at -80 °C until hormonal extraction. The experiment was completely randomized, with three replications and five flasks (10 plants) per repetition. The hormonal quantification was adapted from the protocol described by Fraga *et al.* (2016) and described in the Supplementary file Hormonal analysis methodology.

Statistical analysis: We applied a bootstrap with 1000 replicates to verify the significant difference of gene expression levels. The hormone quantification data were used in a pairwise comparison using t-test ($\alpha = 0.05$); the means were represented in line graphs and a two-factorial analysis was carried out with DPI (0, 3, 5, and 7 DPI) and genotypes, using ANOVA ($\alpha = 0.05$); when significant, the means were separated using a Tukey test ($\alpha = 0.05$). The data were also considered to perform two principal component analyses (PCA), for gene expression and for hormonal content. We performed a collinearity test and variables with significant correlation were discarded from PCA that was performed using normalized data. The two main PCs were presented in a Kernel density estimation (KDE) and plotted in 2D graphic to compare the control *vs.* the inoculated. The statistical analysis ANOVA and Tukey test was performed using R language (R Core Team 2019), the bootstrap analysis and the figures generated using Python 3 language.

Results

Expression analysis: In accordance with the geNorm algorithm (Hellemans *et al.* 2007), *actin* and *ubiquitin* were selected to normalize the expression of the target genes, while *NINJA* was discarded based on the inconsistent results, such as absence of signal amplification and low efficiency.

High covariance was found between *JAZ3*, *MYC2*, *ROMT*, *PR1*, and *STS*. Thus, from these genes only the STS was maintained to perform the PC analysis. The resulting PC1 expressed 33.6 % of the total variation and split mainly inoculated samples from control treatments, while the PC2 expressed 21.3 % of the variation and split genotypes carrying pyramided *Rpv loci* from the others (Fig. 1). The kernel density estimation, built with the PCA data, showed significant gene expression variance among the inoculated treatments, contrasting with the inoculated treatment that presented no significant variation. The genotypes containing pyramided *Rpv* (*Rpv1+Rpv3-1* and *Rpv3-3+Rpv10*) showed greater variation in the PC1, contrasting the *Rpv3-1* and susceptible genotypes that manifested greater variation in the PC2 (Fig. S1 A).

The PCAs (Figs 1 and S1 A) suggest the infection of *P. viticola* induces significant changes in expression of both SA and JA related genes in all genotypes. However, the response of the 'Chardonnay' and 'Regent' were contrasting when compared to the GF15 and 'Bronner' genotypes. The gene expression changes in the genotypes with pyramided *Rpvs* occurred in the early stages and were related with the negative values of the PC2, while, in 'Chardonnay' and 'Regent', the response was later and related with the positive side of the PC2. This result shows that only *Rpv3* did not properly recognize the pathogen.

Regarding the kinetic of the gene expression, *WRKY70* was overexpressed in all genotypes. The strongest induction at 24 HPI occurred in GF15, followed by 'Bronner'. At three and five DPI, 'Regent' showed the stronger expression (Fig. 2). *WRKY70* plays a central role in the jasmonate pathway repression and salicylic pathway activation and can

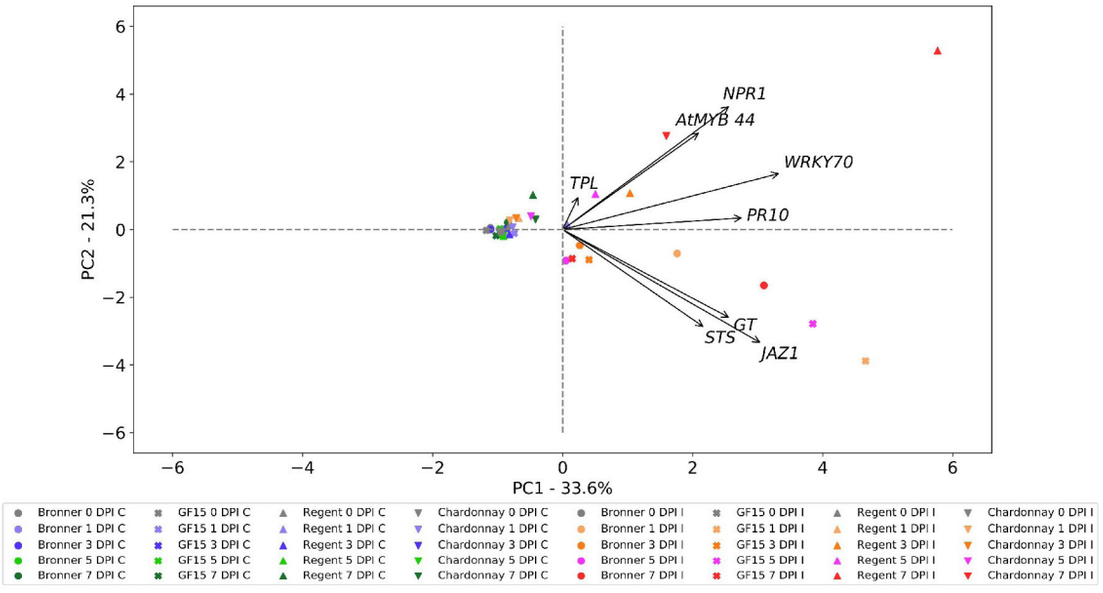

Fig. 1: Principal component analyses based on gene expression data from grapevine leaves inoculated with *Plasmopara viticola* (I) or with water (control, C). The PCA was made with genes from SA-related pathway, Non-expressor of PR1 (NPR1), AtMYB44, WRKY70, JA-related pathway TOPLESS (TPL), Jasmonate Zim-Domain 1 (JAZ1), Pathogen-Related protein 10 (PR10) and Stilbene pathways Stilbene Synthase (STS) and Glycosyltransferase (GT), evaluated on the genotypes 'Bronner' (*Rpv3-3+Rpv10*), GF15 (*Rpv1+Rpv3-1*), 'Regent' (*Rpv3-1*), and 'Chardonnay' (susceptible).

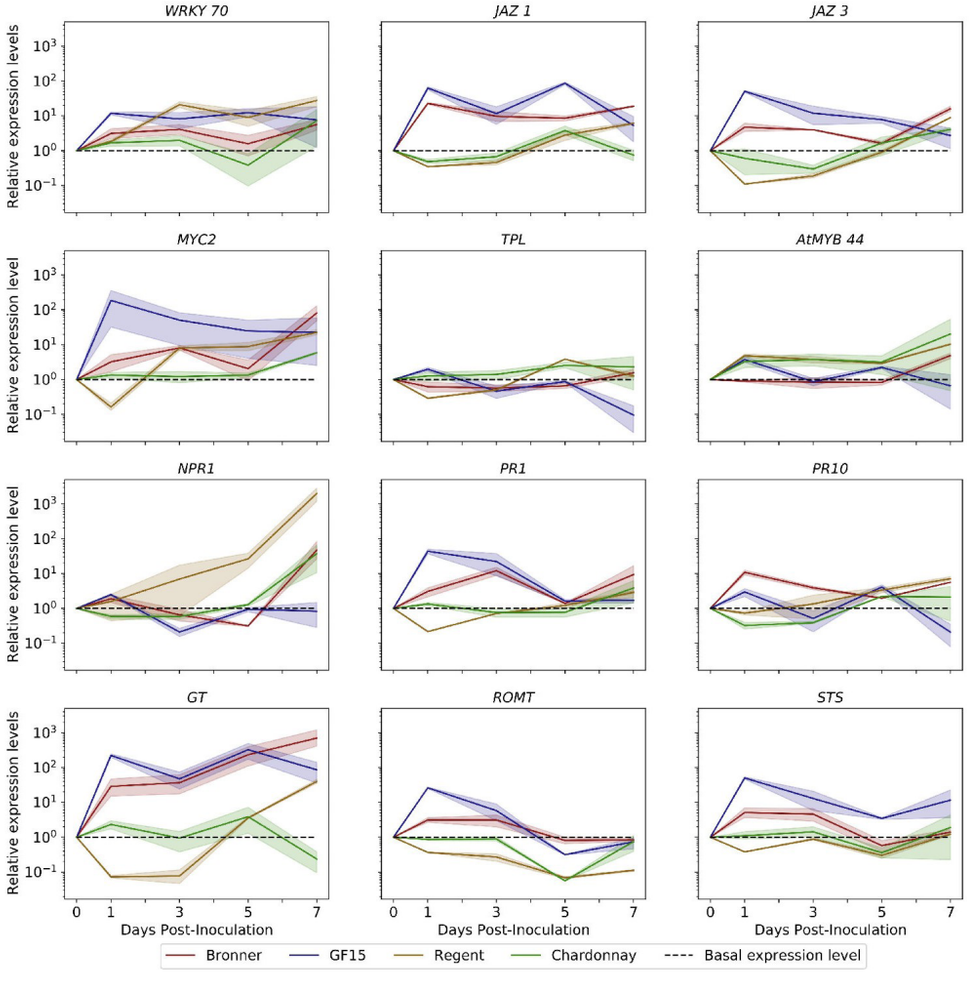

Fig. 2: Kinetics of the fold change in gene expression kinetics of defense pathway genes in grapevine genotypes with different resistance levels. Evaluated genotypes: 'Bronner' (*Rpv3-3+Rpv10*), GF15 (*Rpv1+Rpv3-1*), 'Regent' (*Rpv3-1*) and 'Chardonnay' (susceptible); elicited by inoculation with *Plasmopara viticola*. The shade represents the confidence interval (α=0.05) in a bootstrap analysis with 1000 replications, in the fold change for gene expression in each genotype considering 0 DPI as reference.

be independently activated by *AtMYB44* and *NPR1* (Dong 2004, Shim *et al.* 2013).

In all genotypes, except 'Bronner', *AtMYB44* was upregulated in the first DPI. In the genotypes GF15 and 'Bronner', with pyramided *Rpvs*, *NPR1* was upregulated in the first DPI, while in the susceptible 'Chardonnay' this gene was downregulated. These results pointed to two hypotheses, either *AtMYB44* is the principal pathway to activate *WRKY70* in all genotypes and the activation in 'Bronner' occurred in very early stages of the inoculation, before 24 HPI; or activation of *WRKY70* in the cv. 'Bronner' occurs only by *NPR1*. Additionally, in GF15, both pathways are responsive (Fig. 2).

In both genotypes with pyramided *Rpvs,* the *JAZ* genes were overexpressed from the first DPI and were kept upregulated until the end of the evaluations. The *JAZ1* was downregulated in 'Chardonnay' and 'Regent' from 1 DPI and became overexpressed only at five DPI, and continued to do so at seven DPI in 'Regent'. In 'Chardonnay' and 'Regent', *JAZ3* was upregulated only at seven DPI (Fig. 2). These results show that activation of the JA response occurs only in the genotypes with pyramided *Rpvs*. This fact corroborates the *MYC2* kinetic expression, where the genotypes containing the pyramided *Rpvs* were upregulated from the first until the seventh DPI, while the expression of *MYC2* was induced in 'Chardonnay' only on the seventh day. 'Regent' behaved intriguingly, since *MYC2* expression moved from downregulation status at the first DPI, to overexpression at three DPI. The corepressor *TPL* kinetic expression did not show a clear pattern, as at one DPI in GF15 it was upregulated, while in 'Bronner' and 'Regent' it was downregulated. During the time course, it was overexpressed in 'Regent' and 'Chardonnay' at five DPI and in 'Bronner' only at seven DPI (Fig. 2). The stronger and earlier activation of the defense pathway in the genotypes containing pyramided *Rpv* is evidenced in the expression of the evaluated *PR* genes. From the first until fifth DPI, only GF15 and 'Bronner' overexpressed the *PR1* gene, while for 'Regent' and 'Chardonnay' it occurred only at seven DPI. The *PR10* gene was upregulated in the first DPI in GF15 and 'Bronner'. The gene became overexpressed at five DPI in 'Chardonnay' and 'Regent'.

'Bronner' and Gf-15 also presented a stronger activation of the stilbene pathway. The *STS* was upregulated at all evaluated times in the GF15 and at 1 and 3 DPI in 'Bronner', but it was not overexpressed in the other varieties at any time. The *GT*, responsible for the stilbene glycosylation and piceid synthase, was overexpressed at 24 HPI in all genotypes except 'Regent', which it was upregulated only at five DPI. In the genotypes with pyramided *Rpvs,* the *GT* was strongly upregulated at all evaluated times. Like *STS*, *ROMT* was upregulated at 24 HPI until three DPI only for the genotypes containing pyramided *Rpvs*. While these three genes were downregulated at the first DPI in 'Regent', in the susceptible variety 'Chardonnay' *GT* exhibited a small upregulation at 1 and 5 DPI, and *STS* and *ROMT* were downregulated at 5 DPI (Fig. 2).

Hormonal quantification: Among the target hormones, epibrassinolide (EBL) and gibberellic acid 3 (GA_3) did not reach the limit of detection by the methodology employed. In addition, gibberellic acid 4 (GA_4) was detected only in the untreated genotype containing, *Rpv3-1+Rpv3-2* at three and five DPI, and inoculated *Rpv3-1* at five DPI (Fig. S2 A). In the susceptible genotype (*no Rpv*), the single hormonal change observed occurred at three DPI, with the reduction in the concentration of IAA (Tab. S2, Fig. S2 B). The *Rpv3-3+Rpv10* genotype showed a reduction in ABA concentration at three and five DPI (Fig. 3); behavior similar was observed in the *Rpv3-1* genotype at five DPI, when at the same time, the amount of tZR decreased (Fig. 4). This result contrasts with the presence of a greater amount of tZR, also at five DPI, revealed by the *Rpv3-1+Rpv3-2*. The highest concentrations of SA were observed in the *Rpv3-1+Rpv3-2* genotype at three DPI and in the *Rpv1+Rpv3-1* genotype at seven DPI (Fig. 5). The concentrations of the other hormones JA and Z did not show any significant differences between samples inoculated or not with the *P. viticola* pathogen, in all tested genotypes (Fig. S2 C and D).

Significant positive correlation coefficients were found between the concentrations of SA, tZR and GA_4 and between JA and IAA. Thus, tZR, GA_4 and IAA were discarded from the multivariate analysis. The two PCs shown (Fig. 6 and Fig. S1 B) represent 51.4 % of the total variation; PC1 primarily separated the genotype *Rpv3-1+Rpv3-2* from the others, while PC2 primarily separated, at some points, the inoculated treatment in contrast to the control.

Discussion

The stimulation of both JA and SA pathways reported in this study corraborates other studies developed with *V. vinifera* cited previously (Dufour *et al.* 2013, Li *et al.*

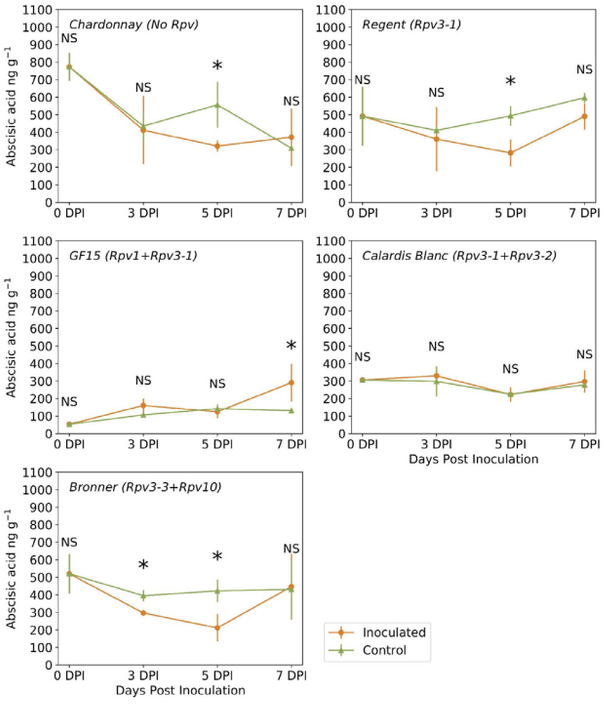

Fig. 3: Kinetics of abscisic acid (ABA) concentration in grape leaf tissue inoculated with *Plasmopara viticola* in genotypes with different levels resistant against downy mildew and susceptible ('Chardonnay'). Pairwise comparison controlled and inoculated conditions, columns with identical letters in the same day are not statistically different by t test (P<0.05).

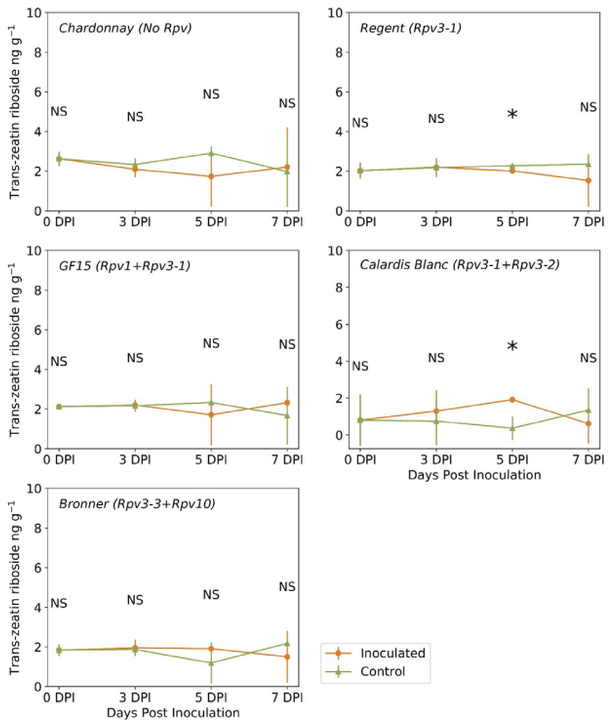

Fig. 4: Kinetics of trans-Zeatin ribose (tZR) concentration in grape leaf tissue inoculated with *Plasmopara viticola* in genotypes with different levels resistant against downy mildew and susceptible ('Chardonnay'). Pairwise comparison controlled and inoculated conditions, columns with identical letters in the same day are not statistically different by t test (P<0.05).

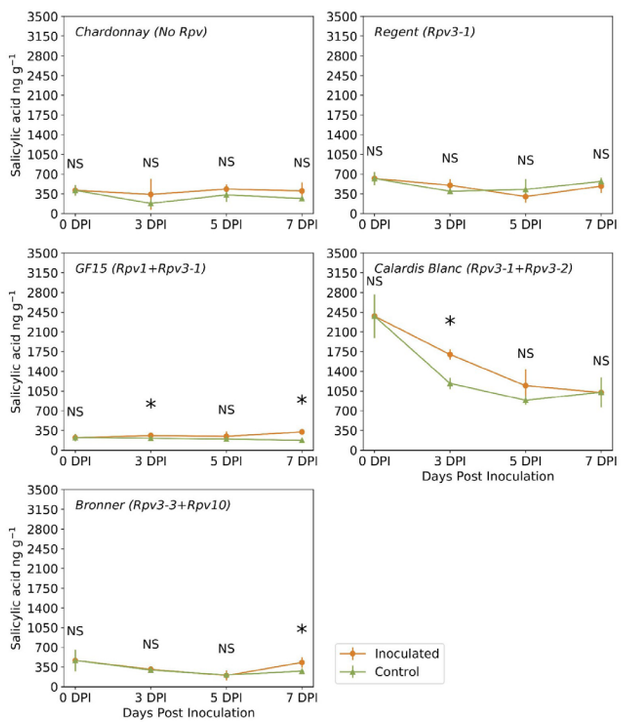

Fig. 5: Kinetics of salicylic acid (SA) concentration in grape leaf tissue inoculated with *Plasmopara viticola* in genotypes with different levels resistant against downy mildew and susceptible ('Chardonnay'). Pairwise comparison controlled and inoculated conditions, columns with identical letters in the same day are not statistically different by t test (P<0.05).

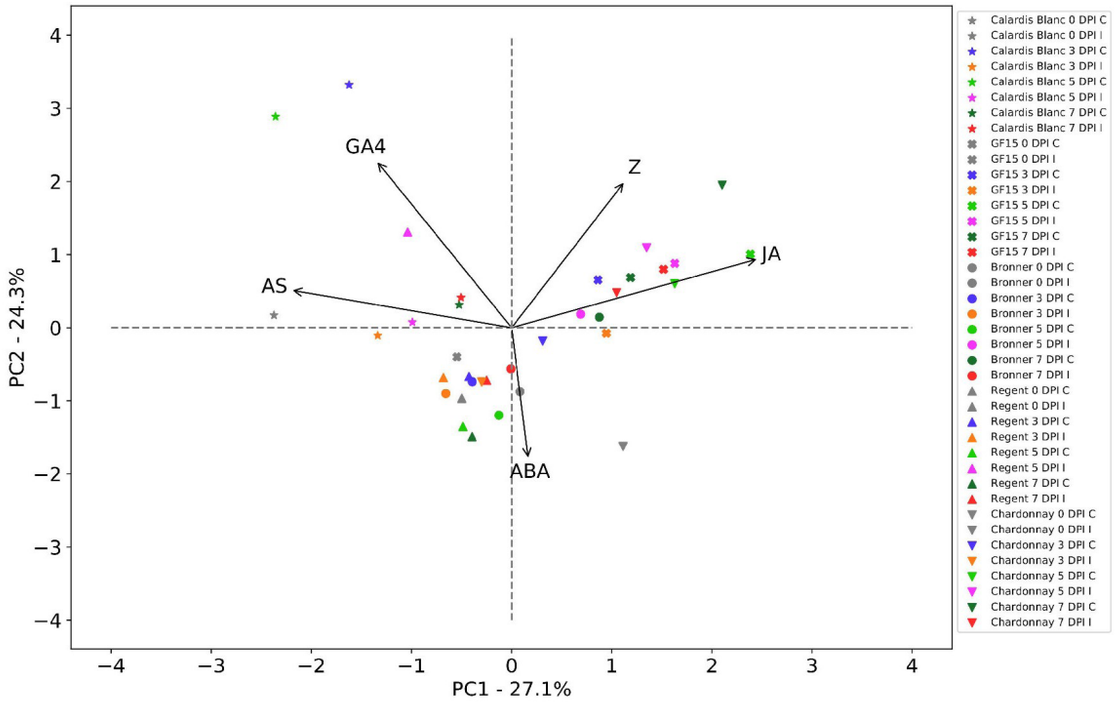

Fig. 6: Principal component analyses based on the concentration of the hormones salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), gibberellic acid 4 (GA4) and zeatin (Z) in grapevine leaves inoculated (I) or control (C) with *Plasmopara viticola* to the genotypes: 'Calardis blanc' (*Rpv3-1+Rpv3-2*), 'Bronner' (*Rpv3-3+Rpv10*), GF15 (*Rpv1+Rpv3-1*), 'Regent' (*Rpv3-1*) and 'Chardonnay' (susceptible).

2015, GUERREIRO *et al.* 2016, Qu *et al.* 2021). The synergistic interaction between JA and SA defense pathways also occurs in other pathosystems (MUR *et al.* 2006). For instance, it was also reported in the pathosystem grapevine x *Erysiphe necator*, another biotrophic pathogen that causes the powdery mildew disease (AGURTO *et al.* 2017). In this case, the synergistic interaction was also verified in genotype containing pyramided resistance genes (*Run1* + *Ren1*).

Since *PR1* is related with the SA signaling pathway, *PR10* is mainly associated with the JA signaling pathway; however, it can also be expressed by other molecules such as the SA (Bari and Jones 2009, Sinha *et al.* 2020). Our results suggest early acting of both SA and JA signaling pathway in the genotypes with pyramided *Rpvs* genes (GF15 and 'Bronner'), while the response of the *Rpv3-1* genotype ('Regent') is close to the susceptible genotype ('Chardonnay') with a late activation of the *PR10* expression (Fig. 2).

SA related genes expression patterns: The SA-related pathway is responsible for the defense system against biotrophic pathogens (Glazebrook 2005, Wasternack and Hause 2013, Ahmad *et al.* 2016), and is also associated with the SAR and ETI, as well as the expression of some *PR* genes, especially *PR1* (Durrant and Dong 2004, Zou *et al.* 2013, Ding *et al.* 2018, Li *et al.* 2019). At the beginning of the signal cascade of the SA-related gene expression induction, there is a functional redundancy to *WRKY70* induction, since this gene may be activated independently by *AtMYB44* or *NPR1* (Dong 2004, Shim *et al.* 2013, Caarls *et al.* 2015, Li *et al.* 2019). *WRKY70* plays a central role in the downstream activation of SA-related genes (Li *et al.* 2019).

Our data show an activation of the SA-related pathway in the first evaluation, with overexpression of the *WRKY70* in all genotypes. However, the pathway to start the activation seems to be influenced by genotype: 'Bronner' did not overexpress *AtMYB44* from 24 HPI until seven DPI, 'Chardonnay' did not overexpress *NPR1* from 24 HPI until five DPI, and only the genotype GF15 overexpressed both genes at 24 HPI.

The kinetic of the *PR1* expression indicates an early activation of the SA-related genes in the genotypes with pyramided *Rpvs* at 24 HPI; however, 'Regent' and 'Chardonnay' overexpressed *PR1* only at seven DPI. The *PR1* expression was previously reported involving the pathosystem *P. viticola* x *V. vinifera*. High *PR1* levels were reported after *P. viticola* inoculation in a susceptible 'Cabernet Sauvignon' (Dufour *et al.* 2013). Moreover, an increase in *PR1* expression at 24 HPI in *V. amurensis* inoculated with the *P. viticola* (Li *et al.* 2015); and in the susceptible 'Shiraz' transgenic with the *MrRPV1* gene, inoculated with *P. viticola* (Qu *et al.* 2021). Even studies using electrical stimulations to enhance the plant defense response in grapevines and *Arabidopsis* report the stimulation of SA-related genes, including the *PR1* (Mori *et al.* 2021). In another study, also developed with 'Regent', an increase in *PR1* expression in the first hours of the infection was reported. Thus, an increase in *PR1* gene expression may have occurred in our experiment before the first evaluation (Guerreiro *et al.* 2016).

JA related genes expression patterns: Based on the covariance observed between genes of JA and SA pathways, as well as, on the gene expression kinetics in general, the results demonstrate the orchestrated involvement of complementary defense pathways activated by the pathogen infection. It is consensus that the JA pathway is involved in the activation of defense response against herbivores and necrotrophic pathogens, whereas the SA pathway is associated with the response against biotrophic and hemi-biotrophic pathogens (Glazebrook 2005, Wasternack and Hause 2013, Ahmad *et al.* 2016). Most studies proved the antagonistic interaction between SA and JA (Aerts *et al.* 2021), and ordinarily, the SA acts to block the JA response (Caarls *et al.* 2015). However, it is also known that this generalist model presents exceptions and additional complexities (Glazebrook 2005, Gimenez-Ibanez and Solano 2013, Li *et al.* 2019). SA can also act in synergy with JA during the infection process in the plant cell defense (Mur *et al.* 2006, Aerts *et al.* 2021), as reported in the acting of the COI1-JAZ2-MYC2 complex, conferring host resistance against biotrophic pathogen through the guard cells closure, without losing resistance against necrotrophic pathogens (Gimenez-Ibanez *et al.* 2017).

As *P. viticola* is a biotrophic pathogen, it could be expected that only the SA defense pathway would be activated. However, the induction of JA related genes was previously reported in resistant grapevine varieties (Guerreiro *et al.* 2016). In the presence of JA-Ile, MeJA, or the JA precursor 12-oxo-phytodienoic acid (OPDA), the F-box protein COI1 - part of the SCF^{COI1} - triggers JA downstream induction of expression of related genes by ubiquitination of JAZ proteins and degradation in the 26S proteasome (Thines *et al.* 2007, Zander 2021). This process of derepression of the MYC2, that physically recruits MED25 (subunit of the Mediator transcriptional co-activator complex), results in the transcription of JA-related genes (Pauwels and Goossens 2011, Zhai *et al.* 2020). Our results demonstrated the activation of *JAZ1* and *JAZ3* in genotypes with pyramided *Rpvs* in the first evaluation times. These results could indicate a replenishment of JAZ levels that were degraded due to the JA-Ile acting (Fig. 7).

The TF *MYC2* has several roles in plant metabolism, being involved in the development of the plant to prepare the host defense (Major *et al.* 2017). Related to the resistance pathway, *MYC2* is repressed by JAZ in the protein level; however, this TF induces *JAZ* expression at the gene transcription level in a fine and complex mechanism of auto-regulation and interaction with other pathways (Aerts *et al.* 2021). In our results, *MYC2* was overexpressed at 24 HPI in the genotypes with pyramided *Rpvs* genes and in three DPI in all resistant genotypes; this kinetic corroborates with the results obtained for the behavior of the *JAZ* genes for these same genotypes. Both the downstream degradation of JAZ by JA-Ile-COI1 action and the release of the co-repressor TPL happen at the same time (Chini *et al.* 2016).

The co-repressor TPL plays important roles in several plant metabolic processes, especially in JA and auxin transcriptional regulation, acting in cell growth and plant immune activation (Plant *et al.* 2021, Powers and Strader 2020). In the absence of JA-Ile, TPL is recruited by JAZ proteins *via* interaction of the NINJA binding protein (Li *et al.* 2019, Pauwels *et al.* 2010). In our results, few changes were reported in the *TPL* gene expression; in 'Regent' and 'Bronner' *TPL* expression was downregulated at 24 HPI, while in GF15 it was upregulated and there were no changes in the susceptible genotype. Throughout the analysis, there is no clear trend of differential expression of TPL between the evaluated genotypes. This fact is probably due to the recycling of NINJA-TPL released by new JAZ protein blocking genes related to JA expression (Li *et al.*

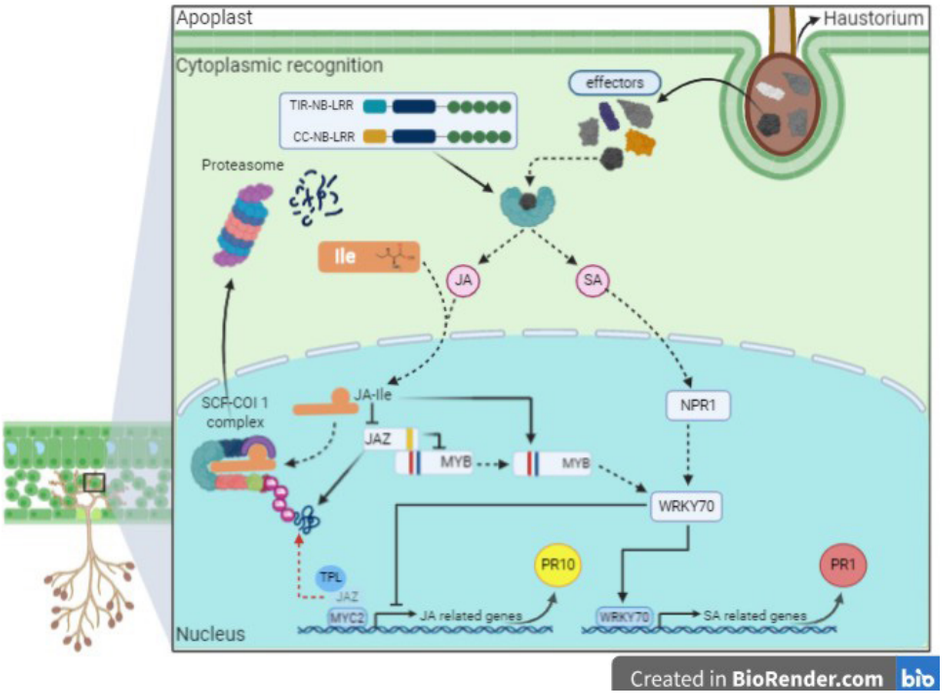

Fig. 7: Proposed model for the interplay between effector triggered immunity activated by JA and SA induced hormones. Pathogen effectors are recognized by Tool/interleukin-1 receptor (TIR) or coiled-coil (CC), nucleotide-binding site (NB) leucine rich repeat (LRR) proteins, activating the salicylic acid (SA) or jasmonate acid (JA) pathway. SA activates the NPR1 that triggers the WRKY70 transcription factor. WRKY70 acts as an antagonist of the JA pathway activating SA related genes, resulting in the PR protein expression, mainly PR1. However, the JA pathway joins with isoleucine (JA-Ile) to activate the SCFCOI1 complex inducing Jasmonate Zim-domain (JAZ) protein ubiquitination and its degradation on the proteasome, inducing the MYB, releasing the *TOPLESS* (TPL), and activating the MYC2, starting the expression of genes related to JA, particularly PR10.

2019). *PR10* expression is a direct product of the JA defense pathway; thus, this gene is commonly used as a marker of JA pathway activation (Guerreiro *et al.* 2016, Yamamoto *et al.* 2018, Martins *et al.* 2021). According to our data, *PR10* was overexpressed at 24 HPI in genotypes containing pyramided *Rpvs*; however, it was only overexpressed in the fifth DPI in the varieties 'Regent' and 'Chardonnay'. The JA- and SA-defense pathways were also reported as activated in the resistant variety 'Regent', grown in a greenhouse, in the first HPI (Guerreiro *et al.* 2016).

Induction of cell resistance, triggered by JA- and SA-defense pathways, was already reported in apples and table grapes (Zhang *et al.* 2017, Zhao *et al.* 2021). Although our results point to this confirmation only in genotypes with pyramided *Rpvs* - GF15 and 'Bronner' - in 'Regent', it may be activated only in the first 24 HPI. This could be due to genetic or epigenetic factors, as this process involves gene transcription activation and a complex modulation of the transcriptome by modifying chromatin and histone configuration (Gómez-Díaz *et al.* 2012, Zhu *et al.* 2016). Therefore, it is additional evidence of the major role that the JA defense pathway plays in this pathosystem.

Stilbene related gene expression patterns: Reports of stilbene involvement in grapevine defense pathways against biotrophic pathogens are common. (Agurto *et al.* 2017, Xu *et al.* 2019, Mori *et al.* 2021, Qu *et al.* 2021). The *STS* is responsible for stilbene biosynthesis, mainly resveratrol, a phytoalexin produced in the cytoplasm, which plays a major role in cellular resistance against pathogens and other abiotic stresses (Zamboni *et al.* 2009). Resveratrol production is stimulated by the JA defense pathway and may be metabolized in other molecules (Vezzulli *et al.* 2007, Ma *et al.* 2019, Martins *et al.* 2021). The resveratrol may be methylated by ROMT, producing pterostilbene, which acts in the suppression of pathogen attacks (Schmidlin *et al.* 2008). The resveratrol may also be glycolyzed by the GT, resulting in piceid production. This process occurs during various grapevine biological processes, such as grape ripening and light stress, and is accumulated during the hypersensitive reactions, reducing mycelial growth and pathogen incidence (Ma *et al.* 2019, Xu *et al.* 2019).

The expression of *STS, ROMT,* and *GT* was strongly induced early in genotypes containing pyramided *Rpv* loci. Intriguingly in 'Regent', these genes were initially down-regulated and only *GT* was upregulated at 5 DPI. Like in SA-related gene expression (Guerreiro *et al.* 2016), the overexpression of these genes in 'Regent' may be occurring in the first hours with no lasting action, as reported for the genotypes with pyramided *Rpvs*. In a study about the transcriptome of genotypes with combinations of *Rpv3* and *Rpv10,* it was reported that the expression of *STS* is the key to stopping *P. viticola* infection after six HPI (Fröbel *et al.* 2019). The same authors also demonstrate that resistant genotypes promote higher levels of *STS* transcription and the regulatory pathway leading to the production of stilbenes differs depending on the resistance locus (Fröbel *et al.* 2019). Thus, the higher levels of stilbene expression in the genotypes with pyramided *Rpvs* reported in our finding can be related to the stronger activation of the resistance ma-

chinery. Some *STS* presented association with the resistance against downy mildew in the hybrid population 'Merxling' x 'Teroldego', carrying the *Rpv3-3* haplotype (Vezzulli *et al.* 2019). In a genetically transformed *V. vinifera* 'Thompson Seedless', in which the *VqSTS6* gene (*STS* gene from *V. quinquangularis*) is overexpressed, there was an increase in the synthesis of resveratrol and trans-resveratrol, and consequently, an increase in the level of resistance to powdery mildew (Cheng *et al.* 2016). An increase of stilbene levels was also reported in genotypes carrying *Run1+Ren1* after being challenged with the biotrophic pathogen *E. necator* (Agurto *et al.* 2017). There was also an increase in stilbenes production in metabolomic studies involving mono-locus, pyramided resistant, and susceptible genotypes and their interaction with *P. viticola* (Ciubotaru *et al.* 2021).

Hormonal patterns: The reduction of the ABA concentration into genotypes containing *Rpv3-1* ('Regent') and *Rpv3-3+Rpv10* ('Bronner'), after mildew inoculation, is possibly linked to the balance of this hormone and the performance of the protein ubiquitination complex (Bueso *et al.* 2014). The action of *RING-type E3 ubiquitin ligase*, a negative regulator of ABA, was verified for grapevine cellular defense against *E. necator* (Wang *et al.* 2017). This pathway is in accordance with what is reported in the ubiquitination process, which involves molecular patterns of pathogen-triggered immunity (PAMP-PTI), resulting in programmed cell death signaling, such as hypersensitivity responses caused by TSI activation, by NBS-LRR genes (Duplan and Rivas 2014, Marino *et al.* 2012).

Nevertheless, IAA plays many roles in the plant metabolism, although controversial results were reported in studies considering plant cell defense. Sometimes IAA is reported as a resistance inductor through systemic acquired resistance (SAR), that results in the induction of quitinases expression that acts as pathogen related (PR) proteins (Van Loon and Van Strien 1999). However, this hormone is also reported as a resistance suppressor while IAA silencing is reported as a PAMP act (Denancé *et al.* 2013, Karasov *et al.* 2017). In our evaluations, IAA concentration was downregulated, only at 3 DPI on the susceptible genotype.

The activation of defense genes linked to the salicylate route was observed in all evaluated genotypes; however, none of these genotypes exhibited an alteration in the kinetics of this hormone quantification (Tab. S2). Only the genotype carrying *Rpv3-1+Rpv3-2* showed significant variation. The early response from the *Rpv3-1+Rpv3-2* genotype can be associated with the potential additive effect obtained from the haplotypes *Rpv3-1* and *Rpv3-2* combination. However, this hypothesis is not supported by the SA kinetics that present a decreasing response over the evaluated times. Our results are in agreement with the behavior of greater resistance induction in the genotypes *Rpv3-1+Rpv3-2* (e.g. 'Calardis Blanc') when compared to *Rpv3-1* genotypes (e.g. 'Regent') in controlled conditions (Eisenmann *et al.* 2019). Yet, until now, this statement is contradictory in field conditions (Zanghelini *et al.* 2019).

Unlike for the other hormones, there is no established knowledge regarding the involvement of Trans-zeatin-riboside in plant defense mechanisms. Nevertheless, a report suggests that this molecule favors the expression of salicylate route genes, promoting the synthesis of *PR1* and increasing resistance in some pathosystems (Choi *et al.* 2011). However, the changes demonstrate that there is possibly an effect due to the genetic background associated with the observed variation. The genotypes carrying different *Rpv3* haplotypes revealed contrasting results. The effect of the genetic background associated with the *Rpv3* genotypes modulating the genetic resistance response to downy mildew is described in the literature (Foria *et al.* 2018). Thus, the genetic background of the genotypes used for this work can be acting on the results obtained, due to the heterozygous nature of the grapevine and the different origins of each *Rpv* loci.

Conclusions

Both SA and JA defense pathways were activated in response to *P. viticola* inoculation. The genotypes containing pyramid *Rpv* genes presented early defense responses. The *Rpv1+Rpv3-1* genotype shows more expressive changes, in both SA and JA metabolic pathways. The stilbene pathway was activated in inoculated plants, demonstrating the involvement of these molecules in the defense pathways for all studied genotypes. The susceptible genotype showed alterations in the expression of genes associated with resistance; however, these are triggered late after the cycle of *P. viticola* causes the damage.

Author Contribution Statement

MDR, RON and MPG conceived and design research; MDR and TCT make the laboratorial and statistical analysis, collect the data and wrote the manuscript; HPS contributed in the hormonal analysis; MS and OT contributed for plant material supplied; MPG; LJW, MS and OT and RON supervised the writing and the data interpretation. All authors read and approved the manuscript.

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