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Differences in defence-related gene expression and metabolite accumulation reveal insights into the resistance of Greek grape wine cultivars to *Botrytis* bunch rot

George T. Tziros[1], Aggeliki Ainalidou[2], Anastasios Samaras[1], Marios Kollaros[2], Katerina Karamanoli[2], Urania Menkissoglu-Spiroudi[3] and George S. Karaoglanidis[1]*

[1] Aristotle University of Thessaloniki, Faculty of Agriculture, Forestry and Natural Environment, Laboratory of Plant Pathology, POB 269, 54124 Thessaloniki, Greece
[2] Aristotle University of Thessaloniki, Faculty of Agriculture, Forestry and Natural Environment, Laboratory of Agricultural Chemistry, 54124 Thessaloniki, Greece
[3] Aristotle University of Thessaloniki, Faculty of Agriculture, Forestry and Natural Environment, Pesticide Science Laboratory, POB 60173, 57001 Thermi, Greece

*correspondence:
gkarao@agro.auth.gr

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ABSTRACT

Berries of 13 Greek grape wine cultivars were evaluated for resistance to *Botrytis* bunch rot. Artificial inoculations on detached berries revealed that the Greek cultivars tested varied regarding their susceptibility to *Botrytis cinerea*. Cultivar (cv.) "Limnio" was found to be highly resistant, while higher susceptibility was observed on berries of cv. "Roditis". To determine the molecular basis of the observed resistance or susceptibility of cv. "Limnio" and "Roditis", an expression analysis of 12 defence-related genes, was carried out on artificially inoculated berries of the two cultivars at different time points after inoculation. Gene expression measurements in the resistant cv. "Limnio" showed that the artificial inoculation with the pathogen triggered the induction of genes encoding pathogenesis-related (*PR*) proteins such as chitinases (*CHIT*), polygalacturonase-inhibiting protein (*PGIP*), serine proteinase inhibitor (*PIN*) and enzymes involved in phytoalexin synthesis such as phenylalanine ammonia-lyase (*PAL*) and stilbene synthase (*STS*). In contrast, on the susceptible cv. "Roditis", most of the same genes were down-regulated. Metabolomic analysis revealed significant differences in the initial metabolic profiles of "Limnio" and "Roditis" berries. Furthermore, in response to inoculation, the abundance of several metabolites increased in the resistant cultivar indicating intensification of metabolic processes. Proline and mannitol accumulation, as well as the modification of metabolites related to phenylpropanoid and lignin biosynthesis, are among the major players in defence responses of the "Limnio" cultivar. The above findings enhance our understanding of the resistance of Greek grape wine cultivars to *B. cinerea* and, at the same time, lay the foundation for breeding wine grape cultivars in the future.

KEYWORDS: *Botrytis cinerea*, grapevine defence, metabolomics, phenylalanine biosynthesis

INTRODUCTION

Greece is one of the oldest wine-producing countries in the world (Koufos *et al.*, 2014). Viticulture occupies an area of about 117,000 ha, of which 67,300 ha yield wine grapes, 21,700 ha table grapes and 28,000 ha raisin grapes (Merkouropoulos *et al.*, 2015). Greek vineyards are characterised by a great number of indigenous grape cultivars, as well as cultivars of eastern origin (Stavrakaki and Biniari, 2016). The National Catalogue of Grapevine cultivars includes 246 different Greek cultivars, of which 210 are wine grapes and 36 are table grapes (http://www.minagric.gr/images/stories/docs/agrotis/Pollaplasiastiko_Yliko/nomothesia_pollaplasiastiko_yliko/2021/ya530_57378_020322.pdf). From the Greek indigenous grapevine cultivars about 200 are cultivated in the country and are characterised by unique oenological and organoleptic properties that provide significant advantages in the grape products market (Lacombe *et al.*, 2011; Kyraleou *et al.*, 2020).

The yield and berry quality of grapevine is limited by a range of biotic and abiotic factors (Li, 2015). Among them, the polyphagous fungus *Botrytis cinerea* causes one of the most serious diseases called *Botrytis* bunch rot, also known as grey mould, in addition to cane and leaf spots (Elad *et al.*, 2016). Annual losses caused by *Botrytis* bunch rot can range from 10 to 44 % (pre- and postharvest), with most losses occurring postharvest (Reglinski *et al.*, 2010). In addition, apart from the yield losses, the pathogen may reduce severely the quality of the produced wine (Ribéreau-Gayon *et al.*, 1998).

The most common method of controlling *B. cinerea* spread in vineyards is by chemical means and approximately 8 % of the global fungicide market is used to control this pathogen (Naegele, 2018; Nishimoto, 2019). However, fungicide use can be harmful to both environment and human health (Droby *et al.*, 2009). Additionally, excessive application of fungicides over the last 50 years has contributed to developing resistant strains of *B. cinerea* in vineyards throughout the world (Hahn, 2014; Panebianco *et al.*, 2015; Rupp *et al.*, 2017). Fungicide use in alternation schemes or reduction in the number of applications can retard, but not prevent, the selection of fungicide-resistant strains or the evolution of resistance and subsequently loss or reduction of fungicide efficacy. Cultural measures could be a viable alternative method of minimising fruit rot damage. Removal of excessive shoots and leaves reduces the formation and spread of conidia and sclerotia (Gubler, 1987). However, cultural control is often unrealistic for large scale commercial farming (Cheung *et al.*, 2020). Thus, the use of *Botrytis*-resistant grape cultivars most likely could complement the chemical practices leading to a more effective crop protection system.

Recently, resistance to *Botrytis* bunch rot in *Vitis vinifera* and other closely related *Vitis* spp., such as *V. labrusca*, *V. lincecumii* and *V. rotundifolia*, has been identified (Naegele, 2018). Disease development depends on various genetic and phenotypic characteristics of the host, such as bunch compactness and morphological, anatomical, or chemical features of the berry skin, which are dependent on the grapevine cultivar (Latorre *et al.*, 2015). Therefore, the development of resistant, high-quality genotypes could reduce the dependence of the viticulture industry on fungicides or other plant disease control measures and could have significant environmental and economic benefits (Rahman *et al.*, 2019a).

Plants have evolved several mechanisms to defend themselves against pathogens (Saigne-Soulard *et al.*, 2015). Constitutive (passive) defences such as structural barriers and pre-formed antifungal compounds help to delay the infection process but are proved to be insufficient (Saigne-Soulard *et al.*, 2015). Induced (active) defence mechanisms leading to the accumulation of antimicrobial compounds such as phytoalexins (stilbenes in *V. vinifera*) and pathogenesis-related (*PR*) proteins have also developed in plants (Adrian *et al.*, 1997; Rivière *et al.*, 2012; Dufour *et al.*, 2013). These active mechanisms are induced only after the plant has recognised an attack by the perception of signal molecules, also called elicitors, which could be abiotic or biotic (Saigne-Soulard *et al.*, 2015). Abiotic elicitors can be physical stimuli like wounding or UV light exposure (Douillet-Breuil *et al.*, 1999; Colas *et al.*, 2012). On the other hand, the term "biotic elicitors" usually refers to molecules secreted by microorganisms derived from the cell walls of fungi, bacteria and host plants (Ebel and Cosio, 1994). *B. cinerea* is one of the most prevalent fungi that damage plants, and in return, plants have developed the ability to recognise their elicitors and to respond strongly to them by inducing defences as in *Vitis* spp. (Langcake and Pryce, 1976).

Although in previous studies, *Vitis* spp. has been evaluated for susceptibility to *Botrytis* bunch rot and defence reactions have been investigated, this is the first study involving Greek grape wine cultivars. Furthermore, only a few researchers report the changes in the metabolic network of grape berries after *B. cinerea* infection and they are focused mainly on secondary metabolites. Hence, the objectives of this study were to a) evaluate 13 Greek wine grape cultivars for resistance against *B. cinerea* by artificial inoculation of grape berries under controlled conditions, b) determine the expression of defence-related genes of the most resistant and susceptible cultivars to *Botrytis* bunch rot and c) elucidate the changes in the metabolic network of the most resistant and susceptible cultivars to *Botrytis* bunch rot.

MATERIALS AND METHODS

1. Sample collection and preparation

Thirteen Greek grape wine cultivars maintained in the experimental vineyard of the Ampelographic Collection, Laboratory of Viticulture, Aristotle University of Thessaloniki, were evaluated for resistance to *B. cinerea* in two consecutive years (2019–2020). The grape wine cultivars chosen for this study were the main Greek wine

grape cultivars that had never been evaluated before for their resistance to *Botrytis* bunch rot.

The vine plants used for sampling were not treated with botryticides during either the 2019 or 2020 growing seasons, while they received regular copper or sulphur applications early in the season for the control of downy and powdery mildew, respectively. Visually healthy bunches were sampled randomly at maturity. Details on the sampled cultivars, their berry skin colour and the sampling date of each year are provided in Table 1. According to the modified E-L system, the experiment was performed using grape berries of the same size and age (harvest stage) and E-L 38 stage (Coombe, 1995). The bunches were harvested from the vineyard and directly transferred to the laboratory for artificial inoculations. Individual berries without blemishes were cut from the rachis of each cultivar, leaving the pedicle attached. Berries were surface disinfected with a 1 % sodium hypochlorite solution for 5 min and thereafter rinsed with distilled water and dried in a laminar airflow cabinet.

2. Pathogen isolate

A virulent single-spore isolate of *B. cinerea*, belonging to the fungal collection of Plant Pathology Lab, AUTh, was used in this study. The isolate was obtained from naturally infected grape berries showing typical grey mould symptoms for the requirements of a study conducted in our lab aiming to determine the genetic variability of *B. cinerea* isolates collected from several hosts in Greece (Samuel *et al.*, 2012).

3. Artificial inoculations and disease assessment

For the artificial inoculations of the grape berries, a previously developed protocol was used (Ciliberti *et al.*, 2015). Briefly, mycelial plugs (4 mm in diameter) were cut from the edges of 4-day-old colonies of the *B. cinerea* isolate, grown onto potato dextrose agar (PDA; Sigma-Aldrich, Germany). Plugs were then placed on the point of rupture of each berry, with the mycelium side touching the berry surface. Grape berries inoculated with PDA plugs without mycelium were kept as a control. Artificially inoculated berries were placed onto plastic racks in plastic boxes (30 × 20 × 10 cm), which contained absorbent paper in their base soaked with sterile water. The boxes were placed in growth chambers at 23°C in the dark for 7 days. For each cultivar, 30 grape berries were used, and the experiment was repeated three times.

On the seventh day after inoculation, grape berries were assessed as healthy or rotten. The index of disease severity was rated on a scale of 0–1 (0: no disease symptom, 1 = berries showing symptoms or signs of *Botrytis* bunch rot), used previously for the evaluation of host resistance to *Botrytis* bunch rot in 27 grape lines (Naegele, 2018). Additionally, the diameter in mm of the spot produced on inoculated grape berries was measured.

4. Plant material for gene expression measurements and metabolite determination

Measurements of resistance levels to *Botrytis* bunch rot of the 13 Greek grapevine cultivars revealed that cv. "Limnio" was the most resistant cultivar, while cv. "Roditis" was the most susceptible one. These two cultivars were selected for defence-related gene expression measurements and metabolite determination after artificial inoculation of the berries with the pathogen. Grape berries of the two cultivars were collected at the harvest stage (E-L 38) in the first year of the experiment (2019) and inoculated with the pathogen, following the same procedure as previously described. Collection of plant material for RNA extraction and metabolite analysis was carried out at 0, 12, 24 and 36 hours post-inoculation (hpi). For each time point, 10 berries were used and the whole experiment was repeated three times. At each time point, all berries were cut in half (two sub-samples), and after removing the seeds but not the skin, they were briefly immersed in liquid nitrogen and kept at -80 °C until further use. The first sub-samples (taken 0, 12, 24 and 36 hpi) were used for RNA extraction, while the second ones (taken 0, 24 and 36 hpi) were analysed for metabolite

TABLE 1. Greek wine grape cultivars used for evaluation of resistance to *Botrytis* bunch rot and dates of sampling.

Cultivar	Berry skin color	Date of sampling (2019)	Date of sampling (2020)
Liatiko	red	8/9/2019	8/19/2020
Robola	white	8/9/2019	8/19/2020
Malagousia	white	8/16/2019	8/19/2020
Kotsifali	red	8/16/2019	8/19/2020
Roditis	white	8/20/2019	8/19/2020
Vidiano	white	8/20/2019	8/19/2020
Agiorgitiko	red	8/20/2019	8/19/2020
Moschofilero	red	8/20/2019	8/19/2020
Assyrtiko	white	8/27/2019	8/19/2020
Savvatiano	white	8/27/2019	8/25/2020
Mandilaria	red	8/28/2019	8/25/2020
Limnio	red	9/2/2019	8/31/2020
Xinomavro	red	9/9/2019	9/4/2020

profiling as the detection of changes in the metabolite profile is expected in a later time point than detection of changes in gene expression of the inoculated berries.

5. Selection of defence-related genes for expression measurements

In total, 10 defence-related genes were selected for expression measurements in the berries of these two cultivars. Among them, genes encoding enzymes implicated in the synthesis of stilbenes (*PAL*, *STS*), genes encoding *PR* proteins (*CHIT1a*, *CHIT3*, *CHIT4c*, *PGIP*, *PIN*) and genes encoding auxin-induced protein 22D (*AUX22D*), jasmonate ZIM domain-containing protein in the TIFY gene family (*VviJAZ2*) and glutathione-S-transferase 25 (*GSTU7*), related to biotic stress responses and modulated in grape *B. cinerea* interaction, were included. In addition, two *B. cinerea* genes (*Bcbot2* and *Bcboa6*), which are required to synthesise phytotoxins associated with grey mould, were also amplified (Supplementary Table 1).

6. RNA preparation

For RNA analysis, each biological sample was comprised of RNA of 10 independent berries (i.e., separate berries were used for each time point) and three technical replicates per treatment. Collected berries were ground to a fine powder using liquid nitrogen and total RNA was extracted from 250 mg of frozen tissue using the Spectrum Total RNA kit (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions. The concentration of the extracted RNA was measured using a P330 nanophotometer (Implen GmbH, Munich, Germany).

7. Quantification of gene expression levels with RT-qPCR

Total RNA, extracted as described above, was used as a template for RT-qPCR. The genes selected for expression measurements and the primers used are listed in Supplementary Table 1. Amplification conditions were 55 °C for 10 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, while the melt curve stage consisted of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The threshold cycle (Ct) was determined using the default threshold settings. The 2-ΔΔCt method was applied to calculate the relative gene expression levels (Livak and Schmittgen, 2001). *Actin* gene (*ACT*; Saigne-Soulard *et al.*, 2015) and the *B. cinerea* β-tubulin gene (*BctubA*; Mamarabadi *et al.*, 2008) were used as endogenous controls and gene expression levels were expressed in a relative manner with respect to those in grape berries inoculated with plugs without mycelium at 0 hpi.

8. Metabolites' extraction, derivatisation, and profiling after gas chromatography–mass spectrometry (GC–MS) analysis

For primary polar metabolite extraction, five replicates (0.5 g each) were analysed. Determination of primary polar metabolites was conducted as described by Ainalidou *et al.* (2016) by GC-MS analysis following derivatisation with methoxy-amine hydrochloride (Sigma Aldrich, St. Louis, USA) in pyridine and N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA reagent; Supelco Bellefonte, USA). Chromatographic separation and identification of metabolites were performed on a Trace GC Ultra-Gas Chromatograph (Thermo Finnigan, San Jose, USA) coupled with a Trace ISQ mass spectrometry detector, a TriPlus RSH autosampler with a split–spitless injector, and an Xcalibur MS platform. One-µl samples were injected with a split ratio of 70:1. GC separations were carried out on a 5 %phenyl-methylsiloxane-fused silica capillary column (TR-5MS 30 m × 0.25 mm × 0.25 µm) with helium as a carrier gas at a flow rate of 1 ml min^{-1}. The peak area integration and chromatogram visualisation were performed using the Xcalibur processing program. For peak identification and mass spectra tic evaluation, the NIST11 database (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used. Mass spectra were cross-referenced with those of authentic standards in the Golm metabolome database (gmd.mpimp-golm.mpg.de; Nardozza *et al.*, 2013; Ainalidou *et al.*, 2016). Quantification of the detected metabolites was based on comparisons with the internal standard adonitol and expressed as relative abundances.

9. Data analysis

Disease incidence for each cultivar was calculated based on the observed symptoms. All data for the three replicate experiments were combined and subjected to analysis of variance (ANOVA) to evaluate the resistance of the 13 Greek grape wine cultivars to *Botrytis* bunch rot. Percentage values were arcsine transformed before statistical analysis. Analysis of variance was performed with SPSS v25.0 (SPSS Inc., Chicago, USA). Significant differences were determined using Tukey's multiple range test at the $P < 0.05$ level. Data of metabolite profiling were subjected to a one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test ($P < 0.05$). The significance level of all hypothesis testing procedures was predetermined at $\alpha = 0.05$.

RESULTS

1. Evaluation of resistance to *Botrytis* bunch rot

Overall, 13 Greek wine grape cultivars were surveyed against *B. cinerea* to evaluate their resistance level. Signs and symptoms observed on inoculated grape berries included rotten brown spots or sunken lesions and surface mycelial growth of *B. cinerea* (Figure 1). Significant differences ($P < 0.05$) were detected among the tested cultivars during both years of the study. For the first year of evaluation (2019), cv. "Limnio" and "Mandilaria" were consistently the most resistant cultivars, considering both measurements of disease incidence and disease severity. On the other hand, cultivars "Vidiano" and "Roditis" were the most susceptible, respectively. Cultivar "Limnio" had the lowest disease incidence, followed by cv. "Xinomavro" and "Mandilaria", although no significant differences were detected among

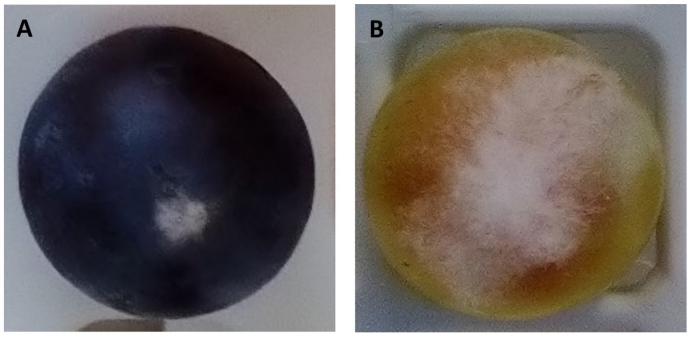


FIGURE 1. Infection of grape berries of the cultivars "Limnio" (A) and "Roditis" (B) seven days after artificial inoculation with *Botrytis cinerea*.

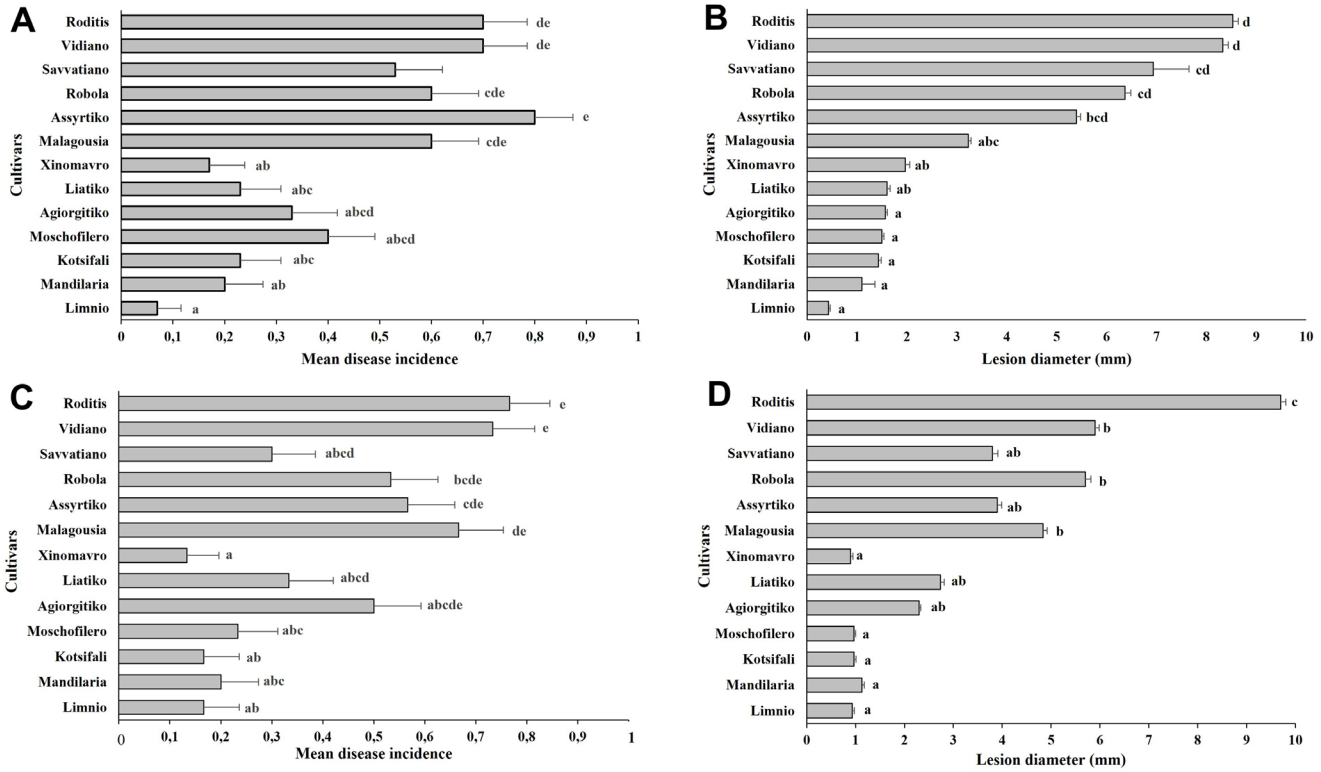


FIGURE 2. Mean disease incidence values (0-1), (A, C) and lesion diameter (mm), (B, D) on berries of different Greek wine grape cultivars, artificially inoculated with *Botrytis cinerea*, 7 days post inoculation in the first (2019), (A, B), and in the second (2020), (C, D), year of evaluation. Different letters on the columns indicate significant differences according to Tukey's Multiple Range Test at $P = 0.05$. Vertical lines indicate the standard error of the mean.

these three cultivars (Figure 2A). In terms of lesion diameter, the smaller lesions were measured on cv. "Limnio" berries, while on cv. "Vidiano" and "Roditis" were observed as the larger lesions (Figure 2B).

In the second year of evaluation (2020), the most resistant Greek cultivar to *Botrytis* bunch rot was "Xinomavro", followed by "Kotsifali" and "Limnio", although there were no significant differences ($P > 0.05$) between these three cultivars (Figure 2C,D). On the contrary, cultivars "Vidiano" and "Roditis" were evaluated as the most susceptible ones (Figure 2C). No significant differences ($P > 0.05$) were detected when evaluating lesion diameter on inoculated grape berries among cvs. "Xinomavro", "Limnio", "Moschofilero", "Kotsifali" and "Mandilaria" on which low disease symptoms were observed. The highest lesion diameter was measured on cv. "Roditis", followed by "Robola" and "Vidiano" with no significant differences ($P > 0.05$) between these two cultivars though (Figure 2D).

2. Defence-genes expression in one resistant and one susceptible cultivar

To determine whether the observed differences in cultivar susceptibility were related to biochemical resistance mechanisms, the expression of 12 defence-related genes was measured in artificially inoculated berries of the most susceptible (cv. "Roditis") and the most resistant

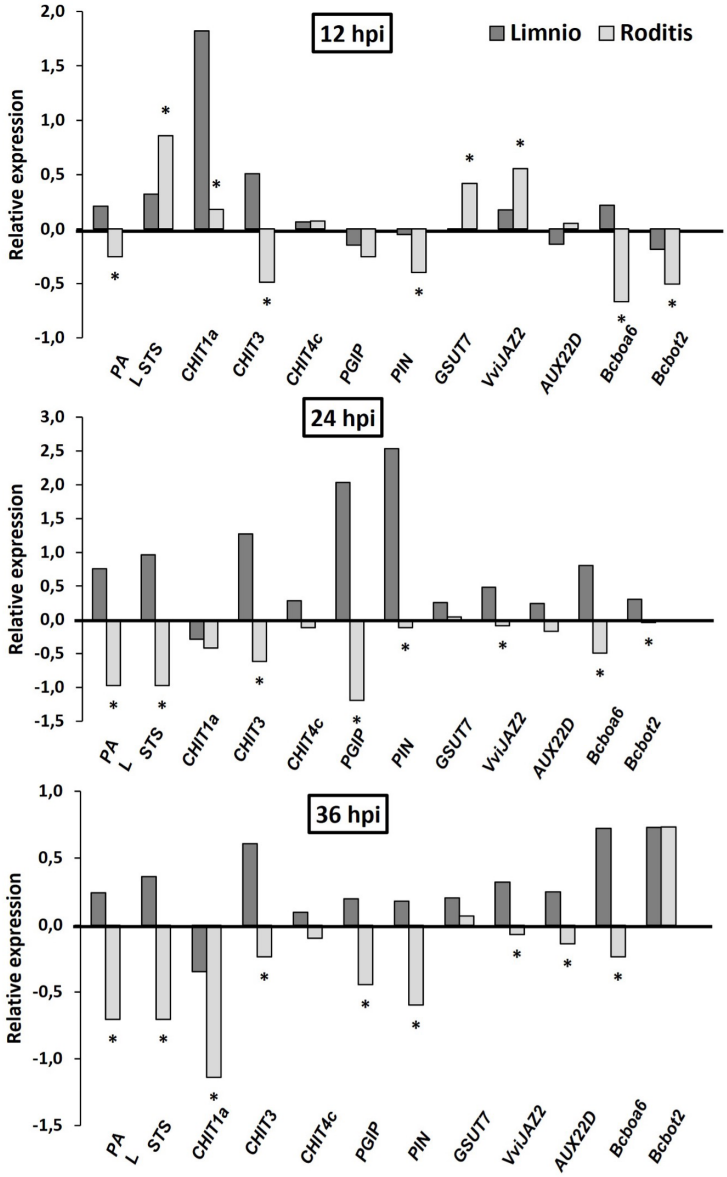


FIGURE 3. Expression analysis of defence-associated genes in grape berries of one resistant (cv. "Limnio") and one susceptible (cv. "Roditis") cultivar by real-time quantitative PCR (RT-qPCR) at 12, 24 and 36 h post inoculation with *Botrytis cinerea*. The y-axis represents relative differences in gene expression compared to that of grape berries before *B. cinerea* inoculation (time point 0 h). *Actin* and β-tubulin gene were used as endogenous controls. Asterisks (*) on the columns indicate statistically significant difference ($P < 0.05$) between the two cultivars within the same gene.

(cv. "Limnio") cultivars. Data on gene-expression measurements are summarised in Figure 3. Expression of phenylalanine ammonia-lyase (*PAL*) and stilbene synthase (*STS*) genes was - induced in the resistant cv. "Limnio", in response to the pathogen. In elicited grape berries, *PAL* and *STS* mRNA accumulation were transient. 24 hpi, the transcription level of *PAL* showed a 4-fold increase, while *STS* expression level was increased by 3-fold. Although these genes were still up-regulated at 36 hpi, their transcription started to decline. On the contrary, in the susceptible cv. "Roditis", *PAL* was down-regulated throughout the three-time points, while *STS*, although up-regulated 12 hpi, was then down-regulated 24 and 36 hours after the inoculation with *B. cinerea*.

The chitinase genes *CHIT1a*, *CHIT3* and *CHIT4c* showed different expression patterns after inoculation with *B. cinerea*. The highest induction level for *CHIT1a* was observed at 12 hpi, and then its expression decreased, exhibiting down-regulation in both cultivars. In the resistant cv. "Limnio", *CHIT3* transcript accumulation started later (24 hpi) and afterwards, it decreased during the susceptible

cv. "Roditis" constantly showed down-regulated expression levels for this gene. On the other hand, *CHIT4c* showed a similar expression pattern to *CHIT3*, as it showed its highest expression at 24 hpi for cv. "Limnio", and it was down-regulated 24 and 36 hpi in cv. "Roditis".

The artificial inoculation of cv. "Limnio" with the pathogen-induced an accumulation of polygalacturonase-inhibiting protein (*PGIP*) gene and that of the inhibitors of serine proteinases (*PIN*). The highest expression was observed at 24 hpi for both genes, showing a significant reduction in the last time point studied (36 hpi).In the susceptible cv. "Roditis", these two genes were constantly down-regulated. The highest reduction was observed at 24 hpi for the *PGIP* gene and 36 hpi for the PIN gene.

The expression profile of the three grapevines and the two *B. cinerea* genes measured by RT-qPCR are also shown in Figure 3. Glutathione-S-transferase 25 (*GSTU7*) and jasmonate ZIM domain-containing protein in the TIFY gene family (*VviJAZ2*) genes were induced in cv. "Limnio", showing their highest up-regulation profile of 24 hpi. Although the auxin-induced protein 22D (*AUX22D*) gene was down-regulated at 12 hpi, it was thereafter up-regulated at 24 and 36 hpi. In contrast, the expression level for these three genes in cv. "Roditis" exhibited a totally different pattern. *GSTU7* gene was up-regulated at the three time points, while *VviJAZ2* and *AUX22D* genes were up-regulated at 12 hpi and then they were down-regulated at 24 and 36 hpi. Surprisingly, the highest induction for the two *B. cinerea* genes (*Bcbot2* and *Bcboa6*) were observed at only 36 hpi for both cultivars (Figure 3).

3. Metabolic responses of grape berries of the most resistant and the most susceptible Greek cultivar

To evaluate the metabolic profile of grape berries after inoculation with *B. cinerea*, metabolites were extracted, and after their derivatisation, they were subjected to GC-MS analysis. By this technique, 50 metabolites were identified and divided into five groups: soluble sugars (16), sugar alcohols (7), organic acids (11), amino acids (11) and other compounds (5) (Figure 4). To detect the changes in the metabolomic profile of the grape berries in response to inoculum, the relative metabolite content between control (0 hpi) and samples at 24 and 36 hpi of the same cultivar is presented in the heat map of Figure 4.

As for the metabolite background of the resistant ("Limnio") and the susceptible cultivar ("Roditis"),

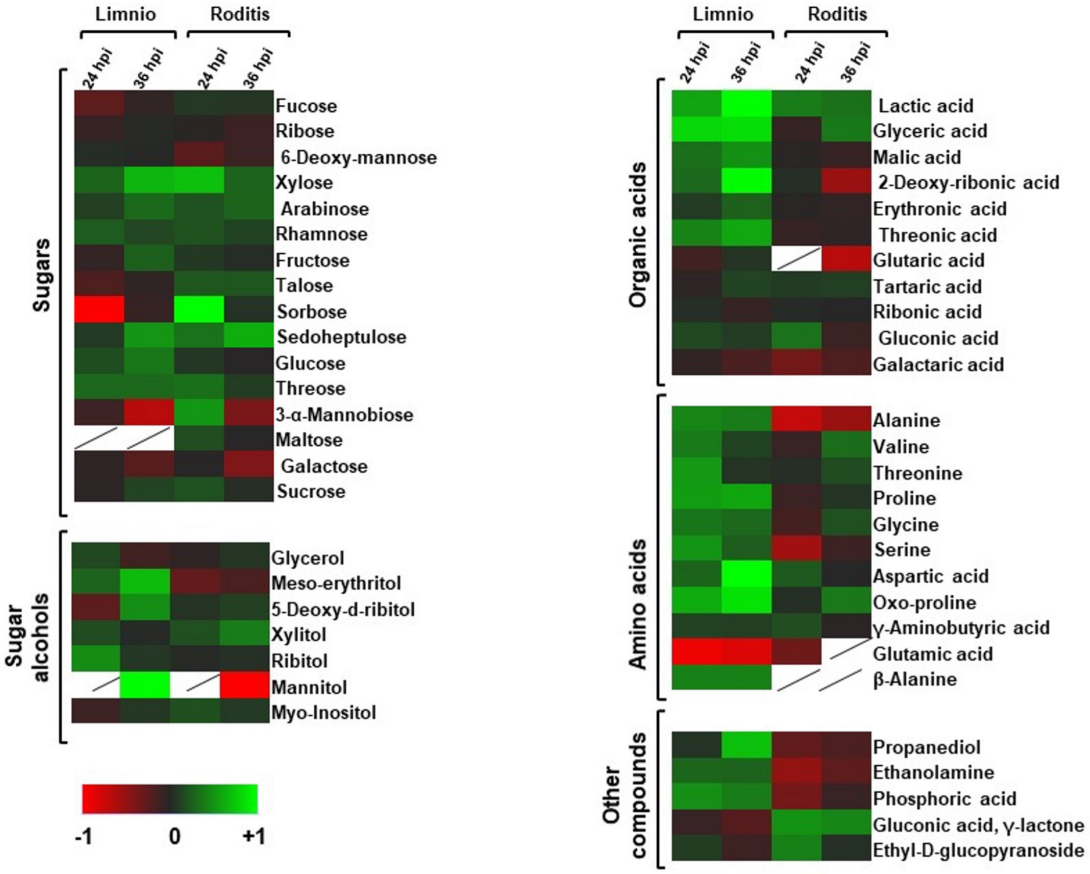


FIGURE 4. Heat map of metabolites indicating changes in grape berries of one resistant (cv. "Limnio") and one susceptible (cv. "Roditis") cultivar at 24 and 36 h post inoculation with *Botrytis cinerea*, compared to the grape berries of the same cultivar before inoculation (0 h post inoculation). Red colour indicates lower, whereas green colour higher relative abundance; the full colour scale is shown at the bottom of the figure; /= non detectable.

the most abundant compounds at 0 hpi were the sugars fructose, glucose, and sucrose and their highest values were recorded in "Roditis" berries (Supplementary Table 2). Several other metabolites were significantly different between "Limnio" and "Roditis". In "Limnio", higher abundance was detected for 11 metabolites, including 5-deoxy-d-ribitol, the acids erythronic, glutaric, gluconic and galactaric, the amino acids valine, proline, glutamic and β-alanine as well as the phosphoric acid and the ethyl-D-glucopyranoside, while in "Roditis" berries aspartic acid, threonine and serine were in excess compared to "Limnio" at the same time point (Supplementary Table 2).

After inoculation, several metabolites were modified in both cultivars in response to *B. cinerea* infection (Figure 4). In particular, *B. cinerea* resulted in the increment of β-alanine and the decrease of 3-α-mannobiose and glutamic acid (Supplementary Table 2). Moreover, glutaric acid decreased at 24 hpi and reverted to the initial level at 36 hpi in both cultivars. On the other hand, "Roditis" and "Limnio" exhibited a reverse trend for meso-erythritol at 24 hpi as this metabolite increased in "Limnio" but decreased in "Roditis" cultivar.

Only in "Limnio" berries the sugars xylose, glucose and fructose increased at 36 hpi. Among sugar alcohols, 5-deoxy-d-ribitol and mannitol accumulated at 36 hpi, as also the organic acids lactic, glyceric, malic, 2-deoxy-ribonic and -erythronic acid. Inoculation with the pathogen also resulted in proline accumulation, as also in the increment of propanediol and phosphoric acid.

The metabolomic profile of "Roditis" berries was modified early after inoculation, at 24 hpi, but the abundance of several metabolites reverted to the initial level of 36 hpi (Figure 4 and Figure 5). In particular, xylose, sorbose, maltose, sucrose, and myo-inositol increased at 24 hpi, while mannitol decreased at the same time point. Furthermore, the higher content of xylitol and lower alanine were recorded at 36 hpi compared to 0 hpi.

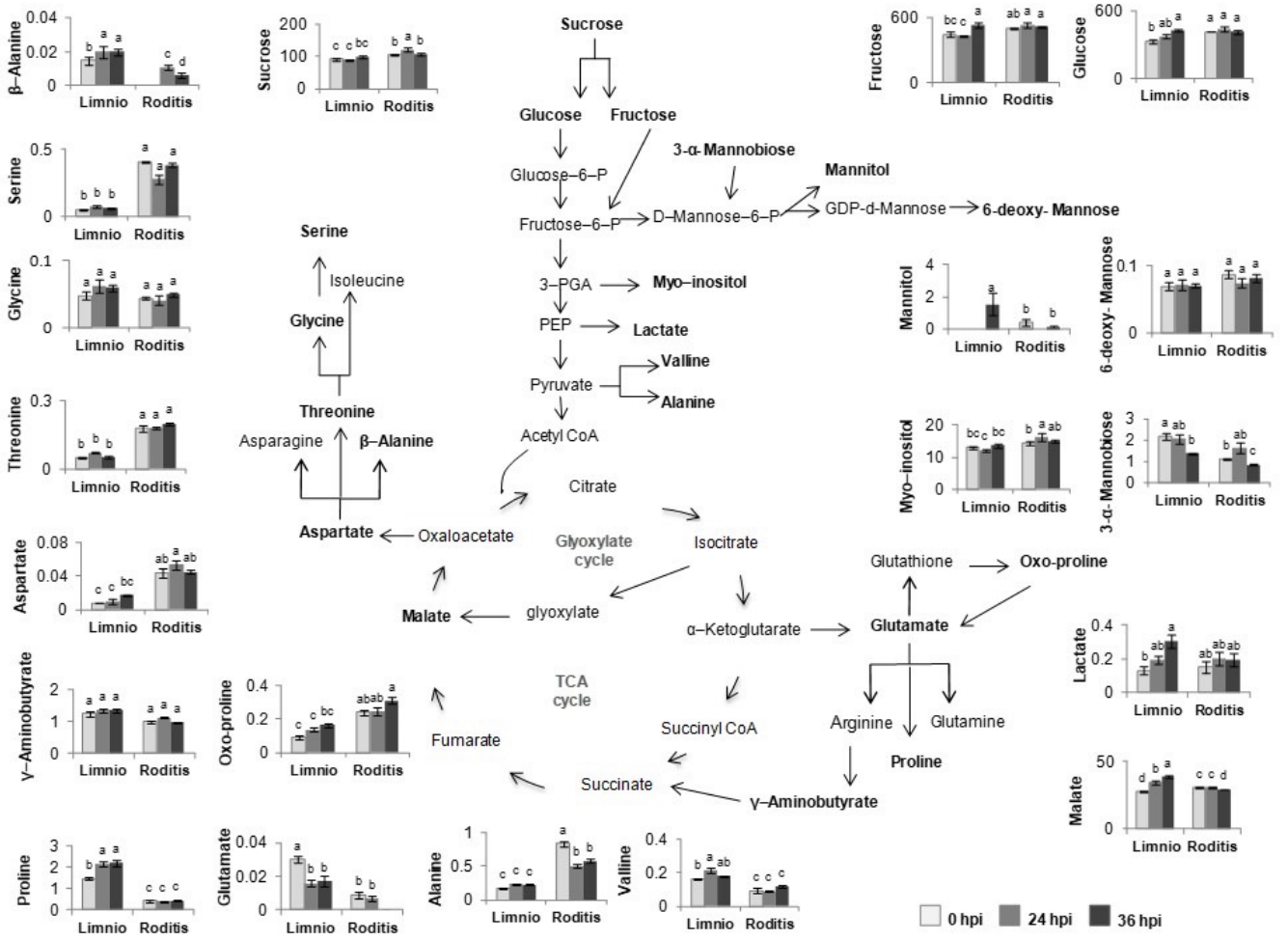


FIGURE 5. Schematic representation of metabolic pathways with metabolites in bold representing those identified in grape berries of one resistant (cv. "Limnio") and one susceptible (cv. "Roditis") cultivar at 0, 24 and 36 h post inoculation with *Botrytis cinerea*. Metabolite quantitative participation is expressed as relative abundance compared to the internal standard adonitol (see supplementary Table 2). Different letters above bars show significant differences between treatments (Duncan's Multiple Range Test; $p < 0.05$).

DISCUSSION

Our study revealed that a high variation existed among the evaluated wine grape cultivars in terms of their susceptibility to *B. cinerea*. Most cultivars were consistent in their response to the pathogen after two cycles of evaluation for two consecutive years. Combining the data obtained from the artificial inoculation of berries (disease incidence and severity), we concluded that "Limnio" was the most resistant cultivar, while "Roditis" was the most susceptible one. To the best of our knowledge, this is the first report documenting differences in *Botrytis* bunch rot susceptibility among different *V. vinifera* cultivars originating from Greece. Most of the widely cultivated *V. vinifera* cultivars are considered susceptible to *B. cinerea*, while other *Vitis* spp. such as *V. rotundifolia*, *V. labrusca*, or other complex hybrids, such as *V. aestivalus* and *V. arizonica,* are moderately to highly resistant (Gabler *et al.*; 2003; Naegele 2018). However, despite the resistance of *V. labrusca* or *V. rotundifolia*-derived lines to *Botrytis* bunch rot, their poor fruit quality makes them undesirable for breeding commercial cultivars (Gabler *et al.*, 2003; Kulakiotu *et al.*, 2004; Naegele, 2018). Similarly, even though *V. vinifera* cultivars are considered, in general, highly susceptible to the pathogen, a certain degree of intraspecific variability exists (Gabler *et al.*, 2003; Wan *et al.*, 2015; Rahman *et al.*, 2019a, Rahman *et al.*, 2019b).

To shed light on the biochemical basis of the observed resistance of cv. "Limnio" or the susceptibility of cv. "Roditis" to *B. cinerea*, berries of the two cultivars were artificially inoculated with the pathogen and the expression of genes encoding enzymes implicated in the synthesis of stilbenes (*PAL*, *STS*) and genes encoding *PR* proteins (*CHIT1a*, *CHIT3*, *CHIT4c*, *PGIP*, *PIN*) were analysed in three different time points post-inoculation (12, 24 and 36 hpi).

Phenylalanine ammonia-lyase (*PAL*), being the first enzyme of the phenylpropanoid pathway, is involved in the biosynthesis of various defence-related compounds (e.g., phenolics, lignin, and salicylic acid) (Saigne-Soulard *et al.*, 2015). In the same pathway, stilbene synthase (*STS*) catalyses the synthesis of resveratrol, which is the principal phytoalexin (Favaron *et al.*, 2009) produced by grapevine in response to biotic or abiotic stresses (Lambert *et al.*, 2013). Further, both *PAL* and *STS* genes showing coordinated gene expression were also induced in leaves infected by *B. cinerea* (Bézier *et al.*, 2002; Belhadj *et al.*, 2008; Lambert *et al.*, 2013), while modification of the plant cell wall by the incorporation of phenolic compounds is an important defence mechanism against this pathogen (Curvers *et al.*, 2010; Bassolino *et al.*, 2013). In our study, expression of *PAL* and *STS* was highly induced in response to *B. cinerea* in the resistant cultivar "Limnio" in all sampling times. In contrast, our results showed that *PAL* and *STS* were rapidly down-regulated in the susceptible cultivar "Roditis". In fact, as activation of phenylpropanoid metabolism is one of the most important resistance reactions observed in plants (Dixon and Paiva, 1995), the variant response of the two cultivars reflects their different resistance to the pathogen.

Pathogenesis-related proteins, glutathione-S-transferase, stilbene synthase and phenylalanine ammonia-lyase were up-regulated in *Vitis vinifera* cv. Trincadeira berries upon infection with *B. cinerea* (Agudelo-Romero *et al.*, 2015). In grapevine, stilbenes such as resveratrol are phytoalexins active against downy and powdery mildew and grey mould (Chang *et al.*, 2011; Malacarne *et al.*, 2011) and the resistance of *Vitis* spp. to *B. cinerea* infection was found to be correlated with *trans*-resveratrol content (Gabler *et al.*, 2003). Additionally, in infected berries, up-regulation of genes involved in the oxidative stress response, such as those encoding glutathione-S-transferase, glutaredoxin, and chitinase class IV, which play a well-known role in abiotic and biotic stresses, was also noticed (Agudelo-Romero *et al.*, 2015).

On the other hand, chitinases play a direct role in plant defence by degrading chitin, a major component of fungal cell walls, and thus inhibit hyphal growth Collinge *et al.*, 1993). Moreover, chitinolytic breakdown products induce the production of phytoalexins and systemic acquired resistance (van Loon and van Strien, 1999). In our study, the chitinase genes *CHIT1a*, *CHIT3*, and *CHIT4c* showed different expression patterns after inoculation with *B. cinerea*. This finding is in accordance with the results of Saigne-Soulard *et al.* (2015), who also noticed different transcript accumulation of these chitinase genes. It is known that although grapevine chitinases are induced by various biotic stresses, their expression level depends on the applied stress, the studied organ, and the grapevine cultivar (Busam *et al.*, 1997; Robert *et al.*, 2002; Aziz *et al.*, 2007).

Polygalacturonase-inhibiting proteins (*PGIPs*) are plant defence proteins that reduce the hydrolytic activity of fungal endopolygalacturonases (*PGs*), preventing, thus, plant cell wall degradation and favouring the accumulation of oligogalacturonides which elicit a variety of defence responses (Rasul *et al.,* 2012). In our study, artificial inoculation of grape berries with *B. cinerea* induced a maximum accumulation of *PGIP* gene at 24 hpi in the resistant cultivar while in the susceptible cv. "Roditis" was observed at its maximum down-regulation at this specific time point. *PGIP* transcript accumulation also exhibited a peak around 24 h in the extract of *B. cinerea*-treated leaves (Saigne-Soulard *et al.*, 2015). *PGIP* and *PIN* have been shown to correlate in several cases with increased resistance of plants to fungi (De Lorenzo and Ferrari, 2002; Akagi *et al.*, 2010; Wang *et al.*, 2013).

Inhibitors of serine proteinases (*PIN*) have potent activity against plant and animal pathogens (van Loon and van Strien, 1999). The RT-qPCR analysis revealed that the *PIN* gene was up-regulated in the berries of the resistant cultivar, in response to fungal inoculation, peaked at 24 hpi and then started downregulating rapidly. A similar pattern of *PIN*

expression was observed in grapevine leaves treated with *B. cinerea* extract, with increased accumulation initiated 5 h after treatment and peaked within 24 h, which then decreased slowly (Saigne-Soulard *et al.*, 2015).

In a recent study, Lovato *et al.* (2019) confirmed their RNA-seq data by analysing the expression profiles of three genes with RT-qPCR. In this study, the induction of *GSTU7* and *VviJAZ2* and the down-regulation of *AUX22D* genes in Garganega berries inoculated with *B. cinerea* were confirmed. However, our results showed that all these three genes were up-regulated in the resistant cv. "Limnio", while the *AUX22D* gene was down-regulated only in the susceptible cv. "Roditis". As expected in a pathogen–host interaction, several genes related to biotic stress responses are modulated, such as genes encoding glutathione-S-transferases (*GSTs*) and TIFY (*VviJAZ2*), which most likely are involved in the berry dehydration response, while auxin pathways are mainly down-regulated (Lovato *et al.*, 2019).

Bcboa6 and *Bcbot2* genes are required to synthesise the two major phytotoxins associated with *B. cinerea*, namely the sesquiterpene botrydial (Colmenares *et al.,* 2002) and the polyketide botcinic acid (Tani *et al.*, 2005; Tani *et al.,* 2006). These two phytotoxins are required to kill host cells and colonise plant tissues (Lovato *et al.*, 2019). For example, a *B. cinerea* double mutant lacking *Bcboa6* and *Bcbot2* showed a significantly reduced virulence on bean leaves compared to the wild-type strain (Dalmais *et al.*, 2011). The fungal genes *Bcboa3*, *Bcboa6* and *Bcboa11*, involved in botcinic acid production, exhibited increased expression during infection of grape berries by *B. cinerea* (Kelloniemi *et al.*, 2015). Interestingly, in our study, *Bcboa6* and *Bcbot2* genes exhibited their maximum transcript levels of 36 hpi in cultivar "Limnio". On the other hand, in the case of the susceptible cv. "Roditis" *Bcboa6* showed down-regulation during the progression of infection, whereas *Bcbot2* was down-regulated at 12 hpi and 24 hpi but showed up-regulation at 36 hpi. The down-regulation of *Bcboa6* and *Bcbot2* was also observed in Müller-Thurgau artificially inoculated berries with *B. cinerea*, pointing out the cultivar-independent suppression of fungal virulence-associated phytotoxin synthesis during noble rot development (Lovato *et al.*, 2019).

Metabolite profiling revealed the initial differences in metabolic processes of the resistant and the susceptible cultivar in the absence of infection, reflecting the differences in morphology and chemical characteristics in the berries of the two cultivars. In particular, a higher abundance of valine, β-alanine, proline, glutamate and 3-α-mannobiose in "Limnio" at 0 hpi and at the same time, high levels of aspartic acid, threonine and serine in "Roditis" were recorded. After artificial inoculation with *B. cinerea,* metabolic modifications were observed in both cultivars. Interestingly, "Roditis" berries metabolism responded early (24 hpi) to *B. cinerea*, but the accumulation in some metabolites was reversed at 36 hpi, indicating the inability of an efficient reaction by the "Roditis" cultivar (supplementary Table 2). This aspect was also evident in the results of the relative expression of the evaluated genes (Figure 3). In particular, at 12 hpi, six genes of "Roditis" were up-regulated compared to the control (0 hpi), and three of them were over-expressed compared to "Limnio", but none of them remained at a high level at 24 hpi. On the other hand, in the "Limnio" cultivar, most of the changes were recorded at 36 hpi Metabolites from all groups, i.e., sugars, organic acids and amino acids were up-regulated in this cultivar, indicating an enhancement of metabolic processes upon infection.

Accumulation of the sugars glucose and fructose in the resistant cultivar revealed the modification of fundamental metabolic pathways, like glycolysis and pentose-phosphate pathway, indicating an immediate and strong response to the pathogen signals (Mutuku and Nose, 2012). Another interesting finding was the accumulation of malic acid in combination with the variations in abundance of derivatives of the TCA cycle, such as aspartic and glutamic acids, which indicate a regulation of the cycle in "Limnio" (Figure 5). Furthermore, aspartic acid and glutamic acid are precursors of other amino-acid biosynthesis affecting the resistance level of each cultivar indirectly. For example, only in the "Limnio" cultivar the decrease of glutamic acid was accompanied by the accumulation of proline, a metabolite that is strongly related to stress conditions of plants by triggering defence responses (Fabro *et al.*, 2004), supporting the importance of glutamic acid catabolism in the exhibition of resistance during pathogenesis in plant tissues (Seifi *et al.*, 2013). Another amino acid that probably contributed to the observed resistance of "Limnio" berries was β-alanine, which was accumulated in infected berries of both cultivars, but its abundance was higher in "Limnio" compared to "Roditis" at all the time points. Previous studies have already indicated the increment of this metabolite under biotic or abiotic stress conditions (Mayer *et al.*, 1990; Broeckling *et al.*, 2005) whilst it is involved in lignin biosynthesis (Broeckling *et al.*, 2005). In this respect, remarkable was the increase of derivatives of the pentose-phosphate pathway as sedoheptulose, erythritol and erythronic acid, indicating the intensification of this metabolic branch, from which erythrose-4-phosphate is derived. It must be mentioned that this metabolite operates as a precursor of the shikimic acid pathway (Santos-Sánchez *et al.*, 2019), known for its further contribution to phenylalanine biosynthesis. Considering also the up-regulation of *PAL* as well as the above-mentioned high levels of β-alanine in "Limnio" berries, we hypothesised that metabolism was activated towards phenylpropanoid and subsequently to lignin biosynthesis as a response to inoculation that is probably related to the resistance of this cultivar. Remarkable was also the modification of mannitol abundance, one of the major metabolites in plant-pathogen interactions (Upadhyay *et al.*, 2015), in "Limnio" berries at 36 hpi. The accumulation of this metabolite illustrates changes in mannitol dehydrogenase activity, an enzyme crucial in mannitol metabolism that belongs to pathogenesis-related proteins (*PR* proteins) (Patel and Williamson, 2016) and is also probably related to the pathogenesis status of the "Limnio" cultivar.

CONCLUSIONS

From the findings of the present study, it is deduced for the first time that indigenous Greek wine grape cultivars exhibit different levels of resistance against *Botryti*s bunch rot. This observation is important as indigenous Greek wine grape cultivars could constitute a valuable genetic resource for future disease resistance breeding programs. Additionally, this differentiation allowed us to elucidate the biochemical basis of resistance in grape berries against the pathogen *B. cinerea*. Genes and metabolite evaluation revealed the induction of plant metabolism, which allowed resistant cultivar to respond strongly and in a timely manner to the pathogen signals by producing signal molecules and activating defence mechanisms such as phenylpropanoids, lignin and *PR* proteins.

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