Virulence-related metabolism is activated in Botrytis cinerea mostly in the interaction with tolerant green grapes that remain largely unaffected in contrast with susceptible green grapes

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Abstract
Botrytis cinerea is responsible for the gray mold disease, severely affecting Vitis vinifera grapevine and hundreds of other economically important crops. However, many mechanisms of this fruit-pathogen interaction remain unknown. The combined analysis of the transcriptome and metabolome of green fruits infected with B. cinerea from susceptible and tolerant genotypes was never performed in in any fleshy fruit, mostly because green fruits are widely accepted to be resistant to this fungus. In this work, peppercorn-sized fruits were infected in the field or mock-treated, and berries were collected at green (EL32) stage from a susceptible (Trincadeira) and a tolerant (Syrah) variety. RNAseq and GC–MS data suggested that Syrah exhibited a pre-activated/basal defense relying on specific signaling pathways, hormonal regulation, namely jasmonate and ethylene metabolisms, and linked to phenylpropanoid metabolism. In addition, putative defensive metabolites such as shikimic, ursolic/oleanolic, and trans-4-hydroxy cinnamic acids, and epigallocatechin were more abundant in Syrah than Trincadeira before infection. On the other hand, Trincadeira underwent relevant metabolic reprogramming upon infection but was unable to contain disease progression. RNA-seq analysis of the fungus in planta revealed an opposite scenario with higher gene expression activity within B. cinerea during infection of the tolerant cultivar and less activity in infected Trincadeira berries. The results suggested an activated virulence state during interaction with the tolerant cultivar without visible disease symptoms. Together, this study brings novel insights related to early infection strategies of B. cinerea and the green berry defense against necrotrophic fungi.

Introduction
Grapevine is one of the most valuable and cultivated crops throughout the world. Most of the cultivars used for wine production are V. vinifera species which were selected due to its organoleptic characteristics. However, this species is highly susceptible to biotic stresses, mostly caused by oomycetes or fungi such as Botrytis cinerea [1, 2]. B. cinerea is a widespread, filamentous, and necrotrophic fungus that infects more than 200 plant species, leading to serious economic losses every year [3, 4]. This pathogen causes grey mold (bunch rot), one of the most severe diseases in grapevines, affecting the yield and quality of production worldwide. As a result, frequent applications of fungicides are needed to protect vineyards, with tremendous economic implications and compromising environmental sustainability [5].

The plant innate immune system (PIIS), a multi-layer and tightly regulated signal transduction component, triggers proteins and metabolites with a defensive role against different pathogens [6]. The PIIS is composed of the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) [7]. PAMPs are recognized by the plant pattern recognition receptors (PPRs), a system of receptor-like kinases or receptor-like proteins, crucial for cell-to-cell communication and extracellular signal sensing [8]. Different examples of PAMPs have been described, such as flg22 (bacterial flagellin), elf18 (bacterial elongation factor-Tu) and, regarding fungi, cell wall polysaccharides, chitin, β-glucans, and ergosterol [9]. For example, the endopolygalacturonases from Botrytis are recognized by the receptor-like protein RBFG1 in Arabidopsis and induce the PTI response, which is not strong but is broad-spectrum immune response [10]. Effector-triggered immunity is the second level of pathogen recognition and requires the perception of pathogen-specific effectors, recognized by plant R proteins and leading to a rapid and robust response [11, 12]. Pathogen recognition by the PTI/ETI systems is followed by a complex signaling network.
that regulates gene expression and the activation of several downstream defense-related pathways, such as the induction of reactive oxygen species (ROS), cell wall modifications to limit fungal growth, or/and the activation of calcium signaling and MAPK cascades resulting in the expression of many defense-related genes and production of secondary metabolites such as phytoalexins [13–15].

The aforementioned plant defenses are shaped by several phytohormones, including salicylic acid (SA), classically associated with resistance against biotrophic pathogens; and jasmonic acid (JA) and ethylene (ET) linked with resistance to necrotrophic fungi, including B. cinerea [15, 16]. Nevertheless, there are several exceptions to this, and the participation of growth and stress-related hormones such as gibberellic and abscisic acid, auxin, and brassinosteroids in plant defense activity has also been described [4, 6]. On the other hand, necrotrophic pathogens have evolved complex strategies to subdue the host immune system. B. cinerea can infect grapevine by mycelium penetration through stomata and wounds or by conidia early invasion, infecting mainly the flower receptacle area and remaining quiescent until berry maturation [17, 18]. In favorable conditions, the conidium develops the appressorium, a specialized infective structure that secretes several phytotoxins and lytic enzymes and promotes an oxidative burst that facilitates host colonization [19]. Nevertheless, in the early stages of infection and before the necrotrophic phase, the fungus can exhibit a short biotrophic behavior that allows the accumulation of biomass and establishment inside the host [20].

Recent studies have been trying to clarify the transcriptome landscape behind B. cinerea virulence in different species, such as cucumber and A. thaliana leaves [6, 21], kiwifruit [22], tomato fruit, and others [23]. Moreover, the fungus transcriptome during infection of grapes (cv. Marselan) was accessed at harvesting stage by microarrays [24], and by RNAseq in cv. Pinot Noir at flowering (EL25/26) [18] and ripening stage [24]. Haile and colleagues [25] also reported the in planta B. cinerea transcriptome during fungus quiescent stage in green hard berries [25]. Nevertheless, all these studies addressed only one cultivar and the mechanisms behind the unusual infection of green berries remain undiscovered. Moreover, the complex host/B. cinerea pathosystem continues to stand poorly understood and more studies are needed, especially due to the extremely plastic transcriptomes of both organisms, which are influenced in a bidirectional manner [6].

Grape clusters can be naturally infected by B. cinerea before bloom and after veraison with increasing susceptibility from veraison to ripening. Between flower and veraison, grape berries are known to be naturally resistant to B. cinerea infection [26, 27]. However, recent studies showed that certain varieties may become infected at green stage when artificial in-field infections are performed [28]. Such is the case of Trincadeira, a very important Portuguese cultivar, which is extremely susceptible to B. cinerea. Additionally, recent studies focused on hormonal metabolism indicated that tolerance against this necrotrophic fungus is mostly based on basal defense, whereas susceptibility is due to delayed defensive responses [16].

In the present work, we compared for the first time the transcriptome and metabolome associated with green hard berries infected with B. cinerea from susceptible (Trincadeira) and tolerant (Syrah) cultivars, bringing innovative insights regarding the early regulatory mechanisms involved in tolerance/susceptibility. Moreover, in planta associated pathogen transcriptomes were analyzed in both grapevine cultivars, disclosing the dynamics of early infection in opposite host scenarios.

Results

Metabolic alterations stimulated B. cinerea infection in both Syrah and Trincadeira grape berries

Green berries are widely recognized as resistant to Botrytis cinerea infection. Notwithstanding, our previous work has shown that green berries of certain cultivars may exhibit heavy symptoms of infection under proper humidity and temperature conditions [26, 28, 29]. In the present work, healthy and infected berries from Trincadeira and Syrah were sampled at green stage (EL32) according to the modified E-L system [30]. Trincadeira extreme susceptibility is thought to be due to high cluster compactness and high growth vigour that creates a low temperature and high humidity microclimate that favours B. cinerea infection [28]. The visual analysis showed that green Trincadeira berries already presented a high level of B. cinerea infection and, in contrast, Syrah showed only mild symptoms with no fungal sporulation being observed (Fig. 1). This was previously confirmed by us for the same samples by qPCR using primers for B. cinerea Polygalacturonase (BcPG1) [16]. This data indicated that the percentage of infection was ~16X fold higher in Trincadeira at green stage than in Syrah. Additionally, though at ripe stage infection symptoms could be observed in this Syrah cultivar they were still less strong than in Trincadeira [16].

To gather insights on how grapes’ metabolism was affected by the infection, GC-EI-TOF/MS was used for the relative quantifications of sugars, organic acids, phenylpropanoids, and other soluble metabolites. Profiling of volatile metabolites was achieved using a GC-EI/QUAD-MS (Supplementary Table S1). Principal component analysis (PCA) was performed with normalized responses (Supplementary Table S1); PC1 and PC2 accounted for 47.70% of the total variability (Supplementary Fig S1) and discriminated samples based on cultivar and infection status, respectively. Within each cultivar, PC2 only established a clear separation between Trincadeira samples, as all Syrah samples were plotted together, revealing a similar metabolic content among control and infected berries. Twenty-five metabolites (23.4% of all detected species) displayed statistically significant differences in abundance between cultivars or between control and infected samples of the same cultivar (Fig 2 and Supplementary Table S1).

Several differences were observed at basal levels when comparing tolerant and susceptible cultivars (Fig 2). Specifically, the phenylpropanoids epigallocatechin and trans-4-hydroxyxycinnamic acid were constitutively present in a larger amount in Syrah, together with shikimic acid, a precursor of phenylpropanoids; those compounds accumulated at higher levels in Syrah than in Trincadeira upon infection. Moreover, the volatile organic compounds (VOCs) 2-ethylfuran, hexanal, (E, E)-2,4-Hexadien-1-al, and phenylacetaldehyde were detected in higher amounts in Syrah than in control Trincadeira. Regarding fatty acids, Syrah showed superior basal levels of hexacosanoic acid (C26) than Trincadeira (Fig 2 and Supplementary Table S1). Very-long-chain fatty acids are required for the biosynthesis of the plant cuticle, generation of sphyngolipids and have been associated with plant defense [31]. Syrah also presented higher levels of a compound from the ursolic/oleanolic acid family than Trincadeira in both basal and under infection conditions, and, in Trincadeira, this lipid increased in response to B. cinerea infection (Supplementary Table S1).

On the other hand, Trincadeira showed higher basal levels of glucose, succinic acid, and 2-oxoglutaric acid than Syrah; additionally, 2-oxoglutaric acid increased in Trincadeira upon infec-
naturally infected with grey mold (observed in infected Trincadeira clusters. (D) Magnification of Trincadeira clusters: fungal sporulation was at developmental stage (EL32). (C) Magnification of Syrah clusters. The metabolism together with fructose, benzaldehyde, and the sterols stigmastanol and campesterol (Fig. 2 and Supplementary Table S1).

Figure 1. Clusters of Vitis vinifera cv. (A) Syrah and (B) Trincadeira grapes naturally infected with grey mold (Botrytis cinerea) at green developmental stage (EL32). (C) Magnification of Syrah clusters. (D) Magnification of Trincadeira clusters: fungal sporulation was observed in infected Trincadeira clusters.

at basal level and might thus be putative markers of tolerance (Figs. 2, 3 and Supplementary Table S1).

RNAseq and functional enrichment analysis indicate a strong transcriptional reprogramming in Trincadeira under infection that was not observed in Syrah

Transcriptional profiling was performed using three biological replicates of control and infected berries of each cultivar. Supplementary Table S2 shows the parsed reads and reads mapped to the predicted transcriptomes, both for B. cinerea and V. vinifera. In our study, the average number of reads uniquely mapped to the grapevine genome was higher in Trincadeira infected samples than in Syrah (19,272,633 and 10,553,075 reads, respectively). On the other hand, B. cinerea average reads in planta were higher in the tolerant infected cultivar (6021 reads, representing 0.057% of the total) than in the susceptible cultivar (2570, representing 0.013% of total reads) (Supplementary Table S2).

The expression of 26,110 different grape genes (87.11% of the total predicted grape genes [33]) and 5478 different B. cinerea genes (44.83% of the total predicted B. cinerea genes [34]) was detected across all samples. Multi-dimensional scaling (MDS) plot of all normalized grape gene counts separated the data into three groups (Supplementary Fig. S2). Similar to the metabolomic results, Syrah samples clustered together independently of the infection status whereas for Trincadeira the MDS plot discriminated infected from control samples.

Differences in gene expression between Trincadeira and Syrah were analyzed comparing the constitutive and under infection transcriptome of both cultivars, and individually for each variety (Infected vs Control) (Supplementary Table S3). The number of differentially expressed genes (DEGs) is reported in Supplementary Table S4. A total of 10,555 grape genes were differentially expressed (FDR < 0.05 and log2 [FC] > 1.0) due to the cultivar (7576 genes) or/and infection status (2979 genes). The Venn diagram illustrates that from the DEGs detected when comparing both infected cultivars, most of them were already detected when comparing the cultivars before infection, implying that the majority of differences are unrelated to specific responses to infection (Supplementary Fig. 3A). The remaining DEGs are likely explained by changes in the Trincadeira transcriptome since there were only 22 DEGs detected in Syrah upon infection (Supplementary Fig. 3B). These results suggest that B. cinerea presence had limited influence on the Syrah transcriptome at the green stage of grape development.

Key biological processes activated or repressed in cultivars (Fig. 4A and Supplementary Table S6) or due to infection (Fig. 4B and Supplementary Table S5) were determined by enrichment analyses of functional categories (P-value < 0.05) using Fatigo [35]. Several functional classes were upregulated in Trincadeira after infection: signaling, carbohydrate-related (including trehalose, starch, and amino sugars metabolism), secondary metabolism (lignin metabolism, stilbenoid, and flavonoid biosynthesis), cell wall-related (xyloglucan modification), stress response (such as biotic and desiccation, oxidative stress), phytoalexin biosynthesis, hormone signaling (mainly ethylene and jasmonate signaling) and lipid metabolism (oxylipin biosynthesis, glycerolipid, and α-linolenic acid metabolism). Moreover, several families of transcription factors were also found to be enriched in the set of genes that was upregulated in Trincadeira under infection (ZIM, WRKY, NAC, MYB, JAZ, ERF, and others). In

Finally, when comparing infected berries, triacontanoic acid, fumaric acid, and the fatty alcohol n-docosan1-ol were detected in higher amounts in Trincadeira upon infection, indicating an acceleration of ripening in susceptible Trincadeira promoted by the fungus B. cinerea as previously reported [28].

In general, B. cinerea infection had little influence on the Syrah metabolome at the green stage. On the other hand, ten metabolites were identified as putative markers of Trincadeira infection, revealing an early metabolic reprogramming upon infection in this susceptible cultivar (Fig. 3 and Supplementary Table S1). Moreover, eight metabolites were more accumulated in Syrah
contrast, an enrichment of the functional classes related to cell wall modification and photosynthesis was observed among the genes downregulated in Trincadeira infected samples (Fig. 4B).

Finally, only three functional categories were enriched in Syrah infected berries. These include, aquaporins, ethylene-mediated signaling pathway, and the ERF subfamily of transcription factors, all of them among the downregulated genes (Fig. 4B and Supplementary Table S5).

**Genes involved in signaling pathways associated with defense are constitutively highly expressed in Syrah whereas in Trincadeira they are activated in response to infection**

Categories of genes encoding R proteins, protein kinases, proteins involved in calcium signaling, ET-mediated signaling, and ZIM, JAZ, and AP2/ERF families of transcription factors are enriched in Syrah when comparing both cultivars at basal level. Expression of genes belonging to these categories was activated in Trincadeira only in response to B. cinerea infection (Fig. 4B and Supplementary Table S5). In detail, B. cinerea infection led to an increase of calcium signaling in Trincadeira, as suggested by the upregulation of many genes involved in calcium-sensing and signaling. Most of those genes were already highly expressed in Syrah when compared to Trincadeira before infection (e.g. calmodulin, calmodulin-binding proteins, and calcium-transporting ATPases) (Table 1). The same holds for the protein kinase functional category, with several up-regulated genes putatively encoding for protein kinases, receptor serine/threonine kinases, and for leucine-rich repeat receptor kinases, which appear to play central roles in signaling during pathogen recognition and plant defense mechanisms (Table 1 and Supplementary Table S3) [36, 37]. Moreover, genes coding for GRAS transcription factors which have been associated with grapevine response to biotic stress [38] were upregulated in Trincadeira under infection and constitutively upregulated in Syrah (Table 1 and Supplementary Table S3).

Finally, the activation of defense-related genes in plants has been associated with different phytohormones, with JA...
Figure 3. Putative positive metabolic markers of B. cinerea infection at green stage (EL32) of development of Vitis vinifera cv Trincadeira and cv Syrah. Metabolites presented were either significantly increased after infection at one or both cultivars (response ratio \( \geq 1.5 \) and p-value \( \leq 0.05 \)) or only identified in infected berries. Square brackets indicate metabolites that were classified only by mass spectral match and grey boxes metabolites that were not detectable.

and ET being essential for plant innate immune system against necrotrophic fungi [4, 39]. Among upregulated genes in Syrah before infection were those involved in ET synthesis and jasmonates’ signaling; these genes were upregulated in Trincadeira after infection (Table 1 and Supplementary Table S3). Previous hormonal profiling performed in the same samples revealed the importance of jasmonates among other hormones in response to B. cinerea during early stages of ripening [16]. This study also validated by qPCR the present RNAseq data regarding hormonal metabolism.

**Basal and under infection primary and secondary metabolism are strikingly different in between tolerant and susceptible cultivars**

Functional enrichment analysis revealed a broad transcriptional contrast between primary and secondary metabolisms of the two cultivars before and under infection. Primary metabolism, in general, was activated in Trincadeira under infection (enrichment in amino sugar, tyrosine, nitrogen, and trehalose metabolisms, glycerolipid catabolism, and \( \alpha \)-linolenic acid metabolism, as well as oxylipin biosynthesis and proteinase inhibitors functional classes) (Table 1 and Supplementary Table S3). Moreover, in Trincadeira, the infection led to the upregulation of genes involved in oxidative stress response, desiccation, temperature, and biotic stress response among others (Table 1 and Supplementary Table S3). On the other hand, cellulose biosynthesis seems to be inhibited in Trincadeira, when comparing both infected cultivars (Table 1 and Supplementary Table S3). Interestingly, transcripts involved in the biosynthesis of the antioxidant \( \alpha \)-tocopherol were upregulated in Syrah at basal level and downregulated in Trincadeira upon infection, in agreement with metabolomics data (Table 1 and Supplementary Table S3).

Concerning the cell wall metabolism, many genes encoding laccases, pectinesterases, and xyloglucan modifications were upregulated in Trincadeira under infection (Table 1). On the other hand, cellulose biosynthesis seems to be activated in Syrah, as suggested by the upregulation of several cellulose synthases when comparing both cultivars before and after infection. Furthermore, carbohydrate metabolism was also affected in Trincadeira by B. cinerea, mainly due to the upregulation of several genes encoding \( \alpha \)‐ and \( \beta \)-amylases and differently expressed genes coding sugar transporters (Table 1 and Supplementary Table S3).

Regarding secondary metabolism, many genes related to alkaloid metabolism and biosynthesis of taxol, terpenoids, and triterpenoids were downregulated in Trincadeira upon infection (Table 1 and Supplementary Table S3). On the other hand, anthocyanin biosynthesis was activated in Syrah pre-infection but triggered by the fungus in Trincadeira, together with stilbenoid
biosynthesis. Finally, phenylpropanoid metabolism was enriched in Syrah when comparing both infected cultivars. (Table 1 and Supplementary Table S3). In general, the data showed several genes involved in secondary metabolism already activated in Syrah at basal level and downregulated in Trincadeira upon B. cinerea infection.
Table 1. Selected grapevine differentially expressed genes in susceptible and tolerant cultivars (FDR ≤ 0.05 and log2 |FC| > 1.5). Complete dataset in Supplementary Table S3

| Unique ID | Syrah Infected/ Syrah Control | Trincadeira Infected/ Trincadeira Control | Syrah Control/ Trincadeira Control | Syrah Infected/ Trincadeira Infected | Functional annotation (Grimplet et al. 2012) |
|-----------|-------------------------------|------------------------------------------|-----------------------------------|--------------------------------------|-------------------------------------------|
|           | log Fold change                |                                          |                                   |                                      |                                           |

Biotic stress response and secondary metabolism

| VIT_00s0266g00070 | 3.34 | 3.16 | 2.61 | Linalool synthase |
| VIT_01s0010g02930 | 4.14 | 3.16 | 5.70 | Calmodulin |
| VIT_03s0038g04390 | 5.89 | 5.70 | 3.24 | Dehydroind 1 |
| VIT_04s0210g00120 | 6.66 | 3.01 | 2.62 | Spermine synthase |
| VIT_05s0020g03200 | 2.04 | 3.41 | 2.52 | Taxadien-5-alpha-ol-O-acetyltransferase |
| VIT_05s0077g01690 | 7.06 | 3.88 | 2.53 | Avr9/Cf-9 rapidly elicited protein 132 |
| VIT_06s0004g05310 | 2.38 | 4.60 | 2.32 | NADPH oxidase |
| VIT_06s0004g07650 | 2.52 | 3.24 | 2.62 | Spermine synthase |
| VIT_09s0002g00220 | 8.83 | 8.73 | 5.19 | Beta-amin synthase |
| VIT_10s0003g03650 | 7.70 | 5.19 | 5.19 | Beta-amin synthase |
| VIT_11s0016g05010 | 4.03 | 4.72 | 4.72 | Lactoylglutathione lyase |
| VIT_12s0034g00130 | 3.76 | 3.27 | 3.27 | Anthocyanidin 3-O-glucosyltransferase |
| VIT_12s0035g01000 | 10.3 | 10.1 | 10.1 | Serine protease inhibitor, serine-type |
| VIT_13s0064g00070 | 6.52 | 6.82 | 6.82 | Cinnamyl alcohol dehydrogenase |
| VIT_14s0068g01920 | 7.33 | 7.91 | 7.91 | Peroxidase |
| VIT_14s0081g00770 | 3.95 | 4.91 | 4.91 | R protein disease resistance protein |
| VIT_15s0039g01280 | 4.47 | 2.82 | 2.82 | Protein kinase |
| VIT_16s0004g00120 | 4.88 | 2.72 | 2.72 | Cationic peroxidase |
| VIT_16s0010g00090 | 6.48 | 6.66 | 6.66 | Resveratrol synthase [V. vinifera] |
| VIT_16s0010g01070 | 6.89 | 6.09 | 6.09 | Stilbene synthase [V. vinifera] |
| VIT_16s0041g00920 | 7.81 | 3.40 | 3.40 | UDP-glucose: anthocyanidin 5-O-glucosyltransferase |
| VIT_18s0117g00370 | 10.7 | 10.8 | 10.8 | R protein L6 |

Oxidative stress

| VIT_04s0008g03600 | 1.68 | 3.77 | Tocopherol cyclase |
| VIT_04s0079g00690 | 11.96 | 11.79 | Glutathione S-transferase 26 |
| VIT_08s0040g00920 | 4.96 | 3.93 | Glutathione S-transferase 25 |
| VIT_10s0003g00390 | 4.04 | 2.25 | Glutaredoxin |
| VIT_16s0039g01410 | 2.12 | 2.81 | Tocopherol O-methyltransferase |

Signaling, Transcription factors and Kinases

| VIT_00s0245g00030 | 7.07 | 3.77 | Receptor serine/threonine kinase |
| VIT_00s0463g00020 | 1.78 | 1.35 | Scarecrow transcription factor 5 (SCL5) |
| VIT_02s0033g00390 | 7.92 | 2.55 | Myb domain protein 113 |
| VIT_06s0004g04990 | 4.81 | CBF transcription factor [V. vinifera] |
| VIT_06s0061g01400 | 5.16 | CBF transcription factor [V. vinifera] |
| VIT_07s0031g01710 | 4.22 | 2.56 | WRKY DNA-binding protein 51 |
| VIT_08s0007g03630 | 2.10 | 2.01 | Calmodulin binding protein |
| VIT_08s0007g08750 | 3.24 | Heat shock transcription factor B3 |
| VIT_08s0032g01220 | 3.16 | 2.49 | Calcium dependent protein kinase 1 |
| VIT_12s0142g00800 | 2.49 | 2.49 | Leucine-rich repeat protein kinase |
| VIT_19s0014g04040 | 6.95 | 2.67 | S-receptor protein kinase |
| VIT_19s0014g04940 | 2.22 | 1.55 | Chinin-inducible gibberellin-responsive protein 1 |

Hormonal metabolism

| VIT_00s0253g000150 | 2.57 | 3.35 | Methyl jasmonate esterase |
| VIT_03s0006g01820 | 4.81 | 7.50 | AOS (allene oxide synthase) |
| VIT_04s0008g02230 | 2.87 | 2.27 | AP2 domain-containing transcription factor ORA47 |
| VIT_04s0008g05760 | 3.17 | 2.68 | WRKY DNA-binding protein 18 |

(Continued)
Table 1. Continued

| Unique ID        | log Fold change | Functional annotation (Grimplet et al. 2012)                          |
|------------------|-----------------|-----------------------------------------------------------------------|
|                  | Syrah Infected/| Trincadeira Infected/ | Syrah Control/ | Trincadeira Control | Syrah Infected/ | Trincadeira Control |
| VIT_05s0049g00510 | 2.33           | −1.97                   | −3.76         | Ethylene response factor ERF1 |
| VIT_09s0029g09140 | −3.66          | 3.77                    | −1.46         | Ethylene-responsive transcription factor ERF003 |
| VIT_10s0003g00590 | 4.31           | 3.02                    | −1.51         | Jasmonate ZIM domain-containing protein 8 |
| VIT_10s0003g03800 | 2.95           | 3.89                    | −5.03         | DREB sub A-5 of ERF/AP2 transcription factor |
| VIT_11s0016g00660 | 5.56           | 2.56                    | −2.27         | AP2 domain-containing transcription factor |
| VIT_11s0016g00670 | 2.13           | 1.80                    | −4.11         | Jasmonate ZIM-domain protein 1 |
| VIT_15s0046g02220 | 7.12           | 3.91                    | −1.51         | ACC synthase |
| VIT_16s0013g00980 | −1.59          | 2.60                    | −5.12         | Ethylene-responsive transcription factor ERF105 |
| **Carbohydrate metabolism** | | | | |
| VIT_00s0181g00180 | −1.71          |                         |              | LHC83 (light-harvesting chlorophyll binding protein 3) |
| VIT_02s0154g00110 | 3.18           | −1.84                   |              | Trehalose-6-phosphate phosphatase (ACP65) |
| VIT_05s0020g03140 | 5.57           | −3.53                   |              | Sugar transporter 13 |
| VIT_05s0077g00840 | 3.60           | −2.19                   | −6.23        | Galactosyl-rhamnose galactosyltransferase |
| VIT_07s0005g01680 | 3.75           | −3.45                   |              | Stachyose synthase |
| VIT_07s0005g02220 | −1.80          | 2.97                    |              | LHCII type I CAB-1 |
| VIT_14s0030g00220 | −2.20          | 2.02                    | 1.81         | Sugar transporter ERD6-like 5 |
| VIT_14s0030g00300 | −2.03          | 2.02                    | 1.81         | Sugar transporter ERD6-like 3 |
| VIT_14s0068g00760 | 4.67           | 1.70                    | −4.73        | Galactosyl synthase |
| VIT_14s0068g00810 | 3.15           | 1.90                    | −3.62        | Raffinose synthase |
| VIT_17s0000g01820 | 3.24           | 1.51                    | −5.12        | Malate synthase, glyoxysomal |
| VIT_17s0119g00150 | 7.65           | 4.01                    | −5.12        | Alpha-aminolevulinic acid/ubiquitin inhibitor |
| **Cell wall metabolism** | | | | |
| VIT_00s02620g00010 | 2.75           | −1.60                   |              | Endo-1,4-beta-glucanase korrigan (KOR) |
| VIT_01s0127g00870 | −1.72          | 2.76                    |              | Polygalacturonase IP630 |
| VIT_01s0137g00240 | −2.39          | 2.76                    |              | Pectin lyase |
| VIT_06s0009g02590 | 11.05          | −11.48                  |              | Pectinesterase family |
| VIT_07s0005g01030 | 2.93           | 2.93                    |              | Cellulose synthase CSLDS5 |
| VIT_08s0007g03330 | 7.19           | −7.61                   |              | Polygalacturonase PG1 |
| VIT_08s0040g01340 | 7.20           | −7.03                   |              | Cellulose synthase CSLA09 |
| VIT_11s0052g01180 | 3.33           | 2.00                    | −2.49        | Xyloglucan endotransglucosylase/hydrolase 23 |
| VIT_12s0059g01010 | 5.66           | 6.27                    |              | Cellulose synthase CSLB04 |
| VIT_18s0122g00690 | 9.65           | −8.42                   |              | Laccase |
| **Lipid metabolism** | | | | |
| VIT_04s0079g00790 | 2.52           | −2.09                   |              | Acyl-CoA synthetases (Acyl-activating enzyme 11) |
| VIT_06s0004g01500 | 2.26           | −3.88                   |              | Lipoxygenase (LOX2) |
| VIT_07s0005g01240 | 3.47           | 1.70                    | −2.40        | Triacylglycerol lipase |
| VIT_07s0141g00060 | 3.68           | 4.80                    |              | Beta-ketoacyl-CoA-synthase |
| VIT_09s0002g01080 | 2.33           | 2.31                    |              | Lipoxygenase |
| VIT_13s0067g01120 | 2.12           | −1.63                   |              | Omega-3 fatty acid desaturase, chloroplast, temperature-sensitive (FAD8) |
| VIT_14s0066g01670 | 6.80           | 6.73                    | −6.63        | Alpha-dioxygenase |
| VIT_16s0022g01120 | 6.73           | 7.56                    |              | Acyl-CoA oxidase ACX3 |
| VIT_16s0022g01150 | 7.56           |                       |              | Acyl-CoA oxidase ACX3 |
Botrytis cinerea presents higher transcriptional reprogramming in the tolerant cultivar

Analysis of RNAseq showed that thousands of reads uniquely mapped to the fungus genome were detected in all infected samples (Supplementary Table S2). Few fungal reads were also detected in all control samples (Supplementary Table S2) confirming the natural and opportunistic presence of *B. cinerea* in the vineyards [40]. Considering in detail the number of *B. cinerea* genes expressed in planta, 531 different genes were Syrah-specific, 166 Trincadeira-specific, and 122 shared by both cultivars (Supplementary Table S7 and S8). PCA plot of all fungus normalized genes counts grouped all the control samples and showed a clear separation between infected cultivars, with the PC1 explaining 71.7% of the variability between control and infected samples (Supplementary Fig. S4).

As a necrotrophic pathogen, *B. cinerea* secrets a broad repertoire of virulence factors, triggering plant chlorosis and host cell death [41]. Several genes associated with virulence and growth were Syrah-specific (Table 2 and Supplementary Table S7). Fungal cell division appears to be promoted in the tolerant cultivar, as suggested by the expression of genes related to the cell cycle, cytoplasmic microtubule and actin cytoskeleton organization, and cellular amino acid biosynthetic process (Fig. 5 and Supplementary Table S7). Furthermore, the results showed a general activation of genes participating in ROS generation and/or oxidation-reduction processes, mainly in Syrah (Fig. 5 and Supplementary Table S7). In particular, the BcNoxR (Bcin03g06840), a major generator of ROS and essential for the development of sclerotia and full virulence [42] (Table 2 and Supplementary Table S7). The same holds true for genes involved in signaling pathways (fungal protein kinases, calcium signaling), protein regulation (e.g. translation, protein folding, and phosphorylation), and protein transport (Table 2 and Supplementary Table S7). Several *B. cinerea* ribosomal proteins involved in translation mechanisms were expressed in both cultivars. Moreover, were expressed mainly in Syrah genes with a putative role in transcription regulation, several transcription factors (e.g. MYB, BZIP, NTO, TFIID, SRF, SFPI1, and CP2, and zinc fingers), and genes involved in chromatin structure and modification (Fig. 5, Table 2, and Supplementary Table S7).

Additionally, many genes associated with fungal cellular degradation processes (e.g. autophagy, proteases, and ubiquitin-dependent protein catabolic process) and fungal cell wall organization (such as several carbohydrate-active enzymes (CAZymes) and chitin synthases) were detected as expressed in Syrah but not in Trincadeira (Fig. 5 and Supplementary Table S7). CAZymes allow plant tissue colonization through host-cell wall modifications and release of carbohydrates for fungus consumption [43, 44]. In detail, 46 and 16 annotated CAZymes were detected as expressed by Botrytis cinerea in Syrah and Trincadeira, respectively (Table 2 and Supplementary Table S7).

Interestingly, several genes with a putative role in carbohydrate conversion were expressed mostly in Syrah berries. This includes, in particular, genes related to glycogen metabolism, glycolytic processes, TCA cycle, and trehalose biosynthesis (Table 2 and Supplementary Table S7). Fungal energy metabolism was also activated in the tolerant cultivar, as suggested by the expression of genes related to ATP synthesis and ATPase activity. Finally, several genes encoding players of lipid and fatty acid metabolism were also mainly expressed in Syrah (Table 2 and Supplementary Table S7) together with fungal major facilitator superfamily (MFS) and sugar transporters. The expression of genes encoding ABC transporters as well as genes involved in the glyoxylate cycle were noticed in both infected grapes (Table 2 and Supplementary Table S7).

On the other hand, some virulence-related fungal genes were also detected in Trincadeira infected berries despite their more advanced state of infection (Fig. 1 and Table 2), namely genes associated with sexual reproduction, fruit body formation, sporulation, and host colonization (Table 2). Moreover, a precursor of riboflavin, lipase 1, and a chitin deacetylase were identified only in Trincadeira and were described as important for *B. cinerea* infection strategy [25, 45].

Discussion

Gray mold is one of the most problematic diseases affecting grapevines [46] and, even though recent studies focus on the molecular basis of *B. cinerea* pathogenicity [22–24], the processes behind necrotrophic infection of fruits at early ripening stages remain uncharted. Moreover, the combined analysis of both susceptible and tolerant green berries towards Botrytis infection has not been performed previously, leaving a gap in the knowledge of the complex and temporal dynamics of *V. vinifera/B. cinerea* pathosystem. Previous to the present study, mechanisms involved in susceptibility of Trincadeira berries were analyzed considering omics approaches [28]. Late green (EL33) and veraison (EL35) berries with grey mold symptoms evidenced a reprogramming of carbohydrate and lipid metabolisms with a putative involvement of jasmonic acid, ethylene, polyamines, and auxins [28]. In this study, we confirmed that this metabolic reprogramming occurs even at an earlier stage of berry ripening (EL32) in the susceptible variety. In contrast, the tolerant Syrah variety remained largely unaffected at early stages. Analysis of the fungal transcriptome indicates that *B. cinerea* is in a more virulent stage of interaction with the tolerant variety, revealing putative new mechanisms associated with this fungal infection.

Pre-activated defenses in Syrah are likely to be responsible for its resilience against Botrytis cinerea attack

Transcriptome and metabolome analyses revealed that Syrah metabolism was only slightly modulated by *Botrytis cinerea* infection, suggesting that tolerance is mainly due to basal defenses. Interestingly, and even though no genes for complete resistance (R genes) to *B. cinerea* have been recognized in plants, functional category enrichment analysis revealed that R proteins and protein kinases were constitutively upregulated in Syrah, while their expression was triggered in Trincadeira by infection. The same was true for genes related to Ca^{2+} mediated signaling, JA signaling pathway, and ET biosynthesis. Calcium signaling modules the regulation of protein kinases, and SA, ET, and JA metabolism [47], which participate in plant response to *B. cinerea* infection [16, 25, 28] and might likewise be important for basal tolerance. Furthermore, plasma membrane Ca^{2+} ATPases appear to be important components of receptor-mediated signaling for plant immune responses and development [48, 49].

The regulation of transcription is known to be paramount for an effective plant defense [50]. Several genes coding for transcription factors (ZIM, JAZ, ERF, AP2, WRKY, NAC) were highly expressed in Syrah healthy berries and upregulated in Trincadeira under infection. Orthologous genes of three transcription factors (WRKY33, BOS1, and MIC2) that influence immune responses in *A. thaliana* [51–53] were also noticed in this interaction and might contribute to the basal resistance of Syrah. The same holds true.
Table 2. Selected *in planta* detected *Botrytis cinerea* transcripts. Genes were only considered present if normalized gene count (RPKM) was equal to zero in all three control replicates and higher than zero in all three infected replicates. Complete dataset in Supplementary Table S7

| Unique ID     |  |  | Syrah Infected |  |  |  |  | Trincadeira Infected |  |  |  |  | Gene Ensembl | Functional annotation |
|---------------|---|---|----------------|---|---|---|---|----------------------|---|---|---|---|---------------|-----------------------|
|               | Inf2 | Inf3 | Inf4 | Inf12 | Inf35 | Inf4 | Inf12 | Inf35 | Inf4 |                   |                       |                       |
| **Virulence and Growth** |       |       |       |       |       |       |       |       |       | Bcphs1          | ER-derived vesicles protein ERV14 |
| Bcin01g01520.1 | 1037 | 949.4 | 409.4 |       |       |       |       |       |       |                   |                       |
| Bcin01g04560.1 | 175.1 | 240.5 | 622.3 |       |       |       |       |       |       |                   |                       |
| Bcin01g05060.1 | 69.1 | 189.7 | 245.4 |       |       |       |       |       |       |                   |                       |
| Bcin01g08260.1 | 451.0 | 619.3 | 267.1 |       |       |       |       |       |       |                   |                       |
| Bcin02g04870   | 822.0 | 376.3 | 324.5 |       |       |       |       |       |       | Bcnb4           | 6,7-dimethyl-8-ribityllumazine synthase |
| Bcin02g08170.1 | 90.7 | 186.9 | 53.7  |       |       |       |       |       |       |                   |                       |
| Bcin02g08280.1 | 100.4 | 137.9 | 237.9 |       |       |       |       |       |       | Bmp1            | Mitogen-activated protein kinase |
| Bcin03g00900.1 | 314.3 | 2158 | 372.3 |       |       |       |       |       |       | Bcsod2          | Superoxide dismutase [Cu-Zn] |
| Bcin03g03880.1 | 301.1 | 206.8 | 356.7 |       |       |       |       |       |       |                   |                       |
| Bcin04g03690.1 | 165.8 | 227.8 | 392.9 |       |       |       |       |       |       |                   |                       |
| Bcin05g00730.1 | 2136 | 2933 | 372.3 |       |       |       |       |       |       |                   |                       |
| Bcin05g01450.1 | 1022 | 468.3 | 1009  |       |       |       |       |       |       |                   |                       |
| Bcin05g05530.1 | 347.3 | 258.5 | 411.3 |       |       |       |       |       |       |                   |                       |
| Bcin05g06320.2 | 3019 | 2759 | 6270  |       |       |       |       |       |       |                   |                       |
| Bcin06g02460.1 | 214.3 | 588.7 | 1015  |       |       |       |       |       |       |                   |                       |
| Bcin07g03340.1 | 384.0 | 527.3 | 454.8 |       |       |       |       |       |       |                   |                       |
| Bcin07g04950.1 | 651.9 | 1342 | 1158  |       |       |       |       |       |       |                   |                       |
| Bcin08g03620.1 | 296.8 | 2530 | 1309  |       |       |       |       |       |       |                   |                       |
| Bcin09g03570.1 | 226.6 | 311.2 | 536.9 |       |       |       |       |       |       |                   |                       |
| Bcin10g00740.1 | 93.2 | 64.0  | 55.2  |       |       |       |       |       |       |                   |                       |
| Bcin10g01180.1 | 555.8 | 763.3 | 658.3 |       |       |       |       |       |       |                   |                       |
| Bcin10g05640.1 | 53.0  | 518.4 | 628.2 |       |       |       |       |       |       |                   |                       |
| Bcin10g07120.1 | 422.1 | 579.6 | 499.9 |       |       |       |       |       |       |                   |                       |
| Bcin11g01720.1 | 1757 | 1689 | 624.5 |       |       |       |       |       |       |                   |                       |
| Bcin11g05430.1 | 825.0 | 679.8 | 1172.6 |       |       |       |       |       |       |                   |                       |
| Bcin11g05640.1 | 243.4 | 394.3 | 576.6 |       |       |       |       |       |       |                   |                       |
| Bcin12g01370.1 | 367.2 | 168.1 | 145.0 |       |       |       |       |       |       |                   |                       |
| Bcin14g01550.1 | 57.3 | 118.0 | 67.9  |       |       |       |       |       |       |                   |                       |
| Bcin15g02590.1 | 146.5 | 201.2 | 173.6 |       |       |       |       |       |       |                   |                       |
| Bcin15g03610.1 | 715.9 | 983.1 | 565.3 |       |       |       |       |       |       |                   |                       |
| Bcin16g03140.1 | 340.6 | 233.9 | 605.1 |       |       |       |       |       |       |                   |                       |
| Bcin17g00930.1 | 691.9 | 950.2 | 819.5 |       |       |       |       |       |       |                   |                       |
| Bcin19g02390.1 | 287.1 | 131.4 | 566.7 |       |       |       |       |       |       |                   |                       |
| Bcin10g04140.1 | 310.6 | 426.5 | 183.9 |       |       |       |       |       |       |                   |                       |
| Bcin11g02950.1 | 81.5  | 559.7 | 96.5  |       |       |       |       |       |       |                   |                       |
| Bcin11g04070.1 | 301.1 | 310.1 | 89.1  |       |       |       |       |       |       |                   |                       |
| Bcin14g03860.1 | 91.0  | 249.9 | 215.5 | 391.0 | 714.4 | 695.8 |       |       |       | Bclme2          | Sporulation protein kinase pit1 |

Continued
Table 2. Continued

| Unique ID          | Syrah Infected | Trincadeira Infected | Gene Ensembl | Functional annotation                          |
|--------------------|----------------|----------------------|--------------|------------------------------------------------|
|                    | RPKMs          |                      |              |                                                |
|                    | Inf2 | Inf3 | Inf4 | Inf12 | Inf35 | Inf4 |                      |                      |                                                |
| Bcin15g03580.1     | 403.5 | 69.3 | 59.7 |       |       |     |                      |                      |                                                |
| Bcin16g01130.1     | 377.5 | 172.8 | 149.1 |       |       |     |                      |                      |                                                |
| **Protein biosynthesis and regulation**                         |                      |                      |              |                                                |
| Bcin02g06250.1     | 824.6 | 377.5 | 325.6 |       |       |     |                      |                      |                                                |
| Bcin02g06900.1     | 716.6 | 2952 | 848.8 |       |       |     |                      |                      |                                                |
| Bcin03g05960.1     | 2454 | 842.8 | 2180.7 |       |       |     |                      |                      |                                                |
| Bcin03g06970.1     | 4723 | 10 790 | 4203 |       |       |     |                      |                      |                                                |
| Bcin07g01170.1     | 3936 | 2703 | 4662 |       |       |     |                      |                      |                                                |
| Bcin12g00420.1     | 444.3 | 305.1 | 1052 |       |       |     |                      |                      |                                                |
| Bcin12g03890.1     | 178.2 | 489.4 | 211.0 |       |       |     |                      |                      |                                                |
| **Carbohydrate metabolism**                                     |                      |                      |              |                                                |
| Bcin01g06740.1     | 97.8 | 268.7 | 115.9 |       |       |     |                      |                      |                                                |
| Bcin01g07270.1     | 230.9 | 317.1 | 547.0 |       |       |     |                      |                      |                                                |
| Bcin01g09950.1     | 330.2 | 113.4 | 97.8 |       |       |     |                      |                      |                                                |
| Bcin02g01650.1     | 492.3 | 225.3 | 194.4 |       |       |     |                      |                      |                                                |
| Bcin02g08340.1     | 538.8 | 739.9 | 638.1 |       |       |     |                      |                      |                                                |
| Bcin07g00940.3     | 112.7 | 51.6 | 44.5 |       |       |     |                      |                      |                                                |
| Bcin08g00740.1     | 649.5 | 446.0 | 769.3 |       |       |     |                      |                      |                                                |
| Bcin09g00150.1     | 415.3 | 380.2 | 983.9 |       |       |     |                      |                      |                                                |
| Bcin10g01500.1     |       |       |       | 1544 | 1411 | 1374 |                      |                      |                                                |
| Bcin11g05700.1     | 835.3 | 382.4 | 989.3 |       |       |     |                      |                      |                                                |
| Bcin12g02300.1     | 986.4 | 1083 | 467.3 |       |       |     |                      |                      |                                                |
| Bcin15g02270.1     | 680.3 | 373.7 | 805.8 | 1169 | 1068 | 1560 |                      |                      |                                                |
| Bcin15g03620.1     | 229.8 | 473.4 | 136.1 |       |       |     |                      |                      |                                                |
| **Lipid metabolism**                                           |                      |                      |              |                                                |
| Bcin01g00440.1     | 212.0 | 194.1 | 83.7 |       |       |     |                      |                      |                                                |
| Bcin02g00210.1     | 230.9 | 317.1 | 547.0 |       |       |     |                      |                      |                                                |
| Bcin04g00760.1     | 301.2 | 413.7 | 178.4 |       |       |     |                      |                      |                                                |
| Bcin10g01780.1     |       |       |       | 1866 | 1705 | 553.6 |                      |                      |                                                |
| Bcin07g09660.1     | 291.3 | 120.0 | 172.5 |       |       |     |                      |                      |                                                |
| Bcin09g02790.1     | 480.4 | 659.7 | 948.3 | 688.0 | 3143 | 3061 |                      |                      |                                                |
| Bcin15g03180.1     | 261.8 | 1078 | 1240 |       |       |     |                      |                      |                                                |
| **Cell Wall metabolism**                                       |                      |                      |              |                                                |
| Bcin01g03390.1     | 62.6 | 85.9 | 222.3 |       |       |     |                      |                      |                                                |
| Bcin01g03790.2     | 46.5 | 63.9 | 55.1 |       |       |     |                      |                      |                                                |
| Bcin02g06930.1     |       |       |       | 555.1 | 760.8 | 247.0 |                      |                      |                                                |
| Bcin03g00640.1     | 1904 | 1664 | 205.1 |       |       |     |                      |                      |                                                |
| Bcin04g01290.1     | 968.6 | 532.0 | 229.4 |       |       |     |                      |                      |                                                |
| Bcin04g03120.1     | 561.4 | 154.2 | 531.9 |       |       |     |                      |                      |                                                |
| Bcin05g07010.1     | 1540 | 705.0 | 3648 |       |       |     |                      |                      |                                                |
| Bcin07g04810.1     | 91.9 | 126.2 | 108.8 |       |       |     |                      |                      |                                                |
| Bcin08g00910.1     | 578.6 | 397.3 | 342.7 |       |       |     |                      |                      |                                                |
| Bcin08g02140.1     | 162.7 | 148.9 | 385.4 |       |       |     |                      |                      |                                                |
| Bcin09g01110.1     | 1051 | 206.3 | 1245.4 | 645.4 | 1769 | 574.3 |                      |                      |                                                |
| Bcin10g02280.1     | 563.6 | 193.5 | 834.5 |       |       |     |                      |                      |                                                |
| Bcin10g05590.1     | 218.6 | 100.1 | 86.3 |       |       |     |                      |                      |                                                |
| Bcin10g06130.1     | 461.6 | 633.9 | 273.4 |       |       |     |                      |                      |                                                |
| Bcin11g04800.1     |       |       |       | 1104 | 2017 | 1965 |                      |                      |                                                |
| Bcin12g05360.1     |       |       |       | 616.7 | 563.5 | 823.1 |                      |                      |                                                |
| Bcin13g04610.1     | 99.7 | 136.9 | 118.1 |       |       |     |                      |                      |                                                |
| Bcin15g00810.1     | 458.3 | 1048 | 542.8 | 656.3 | 599.6 | 2920 |                      |                      |                                                |
for genes belonging to the GRAS family of transcription factors involved in plant response to biotic stress [38].

Omics data underscored a distinct reprogramming of metabolic pathways between cultivars. As previously reported for a more advanced green stage, EL33 [28], the primary metabolism was activated in Trincadeira in response to Botrytis infection albeit photosynthesis appeared to be inhibited. This is a typical response to biotic stress, putatively compensating for activation of defense-related pathways and/or feedback regulation mediated by sugar signals [54] (reviewed by Rojas et al. [54]). Additionally, the expression of genes involved in carbohydrate metabolism and also fatty acid metabolism are known to affect downstream defense responses against fungal pathogens [15, 28, 54]. Transcript and metabolite analyses also indicated that Syrah may better cope with oxidative stress induced by B. cinerea, namely due to accumulation of the antioxidant \( \alpha \)-tocopherol. In fact, a recent study showed that the absence of \( \alpha \)-tocopherol in A. thaliana leaf chloroplasts may delay plant defense activation against B. cinerea through enhanced lipid peroxidation [55].

Transcriptomic data showed that Trincadeira green berries respond to infection by up-regulating genes involved in lipid metabolism (e.g. \( \alpha \)-linolenic acid metabolism). Interestingly, high content in long-chain saturated hexacosanoic acid (cerotic acid) and of a triterpenoid of the ursolic/ oleanolic acid family were observed in Syrah at basal level when compared to Trincadeira. Moreover, genes involved in triterpenoid biosynthesis (coding for \( \beta \)-amyrin synthases) were upregulated in Syrah at basal level. Therefore, the basal tolerance observed in Syrah may rely on the pre-activated lipid-related defenses. Ursolic and oleanolic acids are commonly found in epicuticular waxes of plants and in grapes in particular [56]. These compounds also showed antifungal properties in apple [57] and very-long-chain fatty acids, such as hexacosanoic acid have been associated with plant defense [31].

Many genes involved in phenylpropanoid, and flavonoid pathways were upregulated in Syrah at basal level (Fig. 4 and Table 1). Metabolomic data showed a higher constitutive presence of the phenylpropanoids trans-4-hydroxycinnamic acid and epigallocatechin in Syrah (Fig. 2 and Fig. 3). Epigallocatechin is a precursor of epigallocatechin-3-gallate that is known for its antioxidant properties and has been suggested to promote jasmonic acid signaling in A. thaliana, increasing the resistance to B. cinerea [58].

The putative and positive metabolic markers involved in Syrah basal tolerance also included the volatiles 2-ethylfuran, hexanal, and \((E, E)\)-2,4-Hexadien-1-al. Although studies addressing the role of plant volatiles during necrotrophic infection are scarce, Utto and colleagues (2008) showed that hexanal reduces postharvest infection of tomatoes by B. cinerea [59]. Also, 2-ethylfuran has been reported to prevent downy mildew symptoms in grapevine leaves [60]. Volatiles are indeed involved in resistance to fungal pathogens and they can even contribute to resistance-related phenotypes of neighboring receiver plants [61, 62]. Furthermore, the volatiles benzaldehyde and decanal were accumulated in Trincadeira upon infection and might be used, once validated, as markers of an advanced B. cinerea infection stage.

**Successful defense in Syrah putatively induces wide activation of specific signaling pathways and carbohydrate metabolism in Botrytis cinerea**

The Botrytis cinerea transcriptome in planta was addressed in a few species, such as A. thaliana [5], cucumber [21], kiwifruit [22], tomato, and others [23]. However, the molecular mechanisms associated
with successful B. cinerea infection during the early stages of fruit ripening are unknown. In grapevine, the fungal transcriptome was explored at flowering stage [18], berry mature stage [24, 25], and fungal quiescence on hard green berries [25]. Haile and colleagues (2020) proposed a basal metabolic activity during quiescence with only 289 fungal genes expressed in hard green berries in contrast [25]. Haile and colleagues (Supplementary Table S2).

In general, green fruits are reported as being resistant to infection by B. cinerea, which remains quiescent until the onset of ripening. However, we previously reported green berries of the highly susceptible cultivar Trincadeira exhibiting heavy symptoms of infection [16, 28]. In this study, we confirmed that the fungus is active in Trincadeira but, more surprisingly, it seems highly virulent and far from quiescent in the Syrah cultivar (Fig. 6).

From the 653 B. cinerea genes expressed in Syrah green berries, many were virulence-related (Table 2, Supplemental Tables S7-S8) and only seven genes were in common with the quiescent state described by Hailie et al. [25] (Supplementary Table S9).

Moreover, transcriptomic analysis showed a ratio of 13/1000 and 57/1000 of B. cinerea to V. vinifera reads in Trincadeira and Syrah respectively, indicating a higher transcriptional effort of the necrotrophic fungus to proliferate and overcome Syrah’s basal defenses during infection. The abundance of pathogen transcripts seems to be partially related to fungal biomass and virulence [6, 25, 44]. Our results support the presence of a highly virulent fungus in green fruits of the tolerant cultivar; such high virulence might be related to the natural variation of pathogen strains [41, 43]. Moreover, in Trincadeira green berries only 288 fungal genes were detected, even though symptoms of infection were clear (Fig 1D, Fig. 6). It can be expected that fungal virulence in this cultivar was also higher before the development of symp-toms indicating that the fungus may lose virulence-associated mechanisms when the infection was successful. Similar temporal transcriptional dynamics were observed in the white-rot fungus Obba rivulosa, where a higher level of virulence associated gene expression was detected at early stages of wood colonization, after which the majority of those genes revealed reduced expression [28, 63].

Among the Botrytis cinerea virulence- and growth-related genes expressed in Syrah were noticed chinitin synthases (BcCHSIIIa and BcCHSIV), genes involved in germination (Bc3, Bc and Bmp1), conidia regulators (Bmp3 and Bcsak1), resistance to cyclosporin A (Bcp1) and elicitors of hypersensitive response such as Bcspl1 [64]. Moreover, many genes putatively associated with signaling (e.g. protein phosphorylation and calcium-mediated) and transcription factors were expressed only in Syrah and might be novel and important elements of pathogenesis. Additionally, the expression in Syrah of many genes involved in chromatin structure and modification reenforce the putative association between epigenetic mechanisms and virulence, as previously reported [65].

The fungal transcriptome results suggested that transcriptional activity and protein synthesis were activated during infection especially in Syrah green berries, including the expression of several genes coding for regulators of transcription, ribosomal related, protein folding and protein phosphorylation. Such profile was observed during a quiescent Botrytis cinerea infection and in response to phytoalexins in grapevine [25, 66]. Plants typically trigger an oxidative burst at the early stages of infection, generating several ROS to counteract pathogen invasion. However, as a necrotrophic fungus, B. cinerea can take advantage of that and even produce its own ROS [67]. Several fungal key players in oxidative stress were expressed mainly in Syrah, such as BcNoxR essential for virulence [67], the generator of H2O2 (Bcsod1), per-
Fundamental for early fungus development and host invasion

The association between lipid and carbon metabolisms involving carboxylases, long-chain acyl-CoA synthetase 7, and others. The expression of genes coding for acetyl-CoA dehydrogenase during interaction with the tolerant cultivar, as suggested by lipid and fatty acid metabolisms appear to be active the targeting of glucose into the TCA cycle. Moreover, fungal carbohydrate metabolism was active in Syrah, such active transport is crucial for virulence and typically involved in the growth or exudation of fungal toxins to the intercellular space [6]. Moreover, genes involved in autophagy processes were expressed in Syrah and might have an important role during early infection characterized by nutritional limitations, since it is a process of recycling unnecessary or dysfunctional cellular components with great influence on conidial germination and virulence [71, 72].

During infection, the fungus converts plant hexoses and fructose into mannitol, which together with trehalose, are the most common fungal storage carbohydrates. Interestingly, fungal carbohydrate metabolism was active in Syrah, as suggested by the expression of genes involved in glycolgen metabolism, glycolytic processes, and trehalose biosynthesis. Similar results were described during sunflower infection [73] and suggests the targeting of glucose into the TCA cycle. Moreover, fungal lipid and fatty acid metabolisms appear to be active in planta during interaction with the tolerant cultivar, as suggested by the expression of genes coding for of acetyl-CoA dehydrogenase and carboxylases, long-chain acyl-CoA synthetase 7, and others. The association between lipid and carbon metabolisms involving glyoxylate and TCA cycles was previously hypothesized as fundamental for early fungus development and host invasion before having access to host nutrients [74]. On the other hand, several genes associated with gluconeogenesis (e.g. pyruvate carboxylase, a pyruvate kinase, two glucose-6-phosphate, and others) were expressed only in Trincadeira and are likely associated with fungal proliferation after successful infection. Since B. cinerea was only capable of successful infection in Trincadeira, these results may be important to understand the dynamics of proliferation and infection strategies of the fungus. In particular, they provide insights into how fungal and plant carbohydrate metabolisms are balanced with both fungal and plant defensive strategies.

A comparative meta-analysis was performed in order to retrieve differences between our data and the Botrytis cinerea transcriptome during infection of ripening berries as described by Haile et al. (2020) [25]. This study integrated transcriptome and metabolome data to investigate the crosstalk between the plant and the fungus during pathogen quiescence and egression. It is noteworthy to mention the limitations of comparing both studies since different pathogenic infection stages were considered. Nevertheless, the comparison revealed a broad repertoire of expressed transcripts (Supplemental Table S9), which is indicative of high genome plasticity and transcriptional flexibility [6, 67, 75]. In fact, this plasticity may contribute to the aptitude of Botrytis cinerea to infect a wide number of plant species. Common functional classes were also observed among the expressed genes, such as redox processes, ATP-related, or protein folding classes. In both studies, several genes coding for ribosomal proteins were also expressed suggesting that ribosomes and translation may play a fundamental function in the infection process. Plant ribosomal proteins have been recently associated with biotic stress responses [76], but their role in pathogenesis remains intriguing. Moreover, an uncharacterized secreted protein (Bcin15g00100) was expressed in all the samples analyzed in both studies, deserving special attention since the orthologous in Blumeria graminis was associated with virulence [77].

Conclusions

Understanding grey mold disease processes may disclose new and efficient management strategies. Our study revealed a contrast response between Syrah and Trincadeira cultivars, underlining the importance of studying cultivars with different susceptibility/tolerance levels and specifically at a stage that is generally thought to be low or even non-susceptible. Syrah was barely affected by B. cinerea infection at the green stage, eventually due to pre-activated defensive mechanisms involving specific signaling pathways, hormonal regulation and secondary metabolism. In contrast, Trincadeira was severely affected by B. cinerea and reprogrammed primary and secondary metabolisms, putatively regulated by jasmonate- and ethylene- mediated signaling pathways and transcription factors of the ZIM/JAZ, NAC, MYB, ERF and GRAS families. This study also suggested promising metabolic markers of tolerance against grey mold disease at early stages, including 2-ethylfurane, hexanal, (E, E)-2,4-Hexadien-1-αl, cinnamic acid, shikimic acid, hexacosanoic acid, phenylacetaldehyde, and epigallocatechin.

An opposite scenario was found in the fungus with higher transcriptional activity shown in the tolerant cultivar. The study put in evidence the plasticity of the pathogen’s transcriptome, revealing several genes related to virulence and fungal growth, signaling, carbohydrate and lipid metabolism expressed during infection of Syrah green berries, which might be important for early stages of infection. Nevertheless, different genes related to growth and virulence were also detected only in Trincadeira and might be important to understand regulatory mechanisms behind necrotrophic fungus proliferation after successful infection. Since only few genes have been described previously to be involved in pathogenicity [67], the newly identified putative elements of virulence might be targeted for functional characterization and to develop efficient control strategies. Botrytis cinerea infects several plant species worldwide and the knowledge gathered in this in vivo pathogen interaction study may provide valuable hints to be translated to other plant species.

Materials & methods

Plant material and fruit inoculation

Contaminated grapevine plants were the source to isolate B. cinerea which was maintained at 5°C in potato dextrose agar (Difco, Detroit, MI, USA). Green berry clusters (Trincadeira and Syrah) were sprayed with a conidial suspension EL29 [28] and collected after visual monitoring of symptoms at green stage EL32 (modified E-L system [30]). Control clusters were sprayed...

A comparative meta-analysis was performed in order to retrieve differences between our data and the Botrytis cinerea transcriptome during infection of ripening berries as described by Haile et al. (2020) [25]. This study integrated transcriptome and metabolome data to investigate the crosstalk between the plant and the fungus during pathogen quiescence and egression. It is noteworthy to mention the limitations of comparing both studies since different pathogenic infection stages were considered. Nevertheless, the comparison revealed a broad repertoire of expressed transcripts (Supplemental Table S9), which is indicative of high genome plasticity and transcriptional flexibility [6, 67, 75]. In fact, this plasticity may contribute to the aptitude of Botrytis cinerea to infect a wide number of plant species. Common functional classes were also observed among the expressed genes, such as redox processes, ATP-related, or protein folding classes. In both studies, several genes coding for ribosomal proteins were also expressed suggesting that ribosomes and translation may play a fundamental function in the infection process. Plant ribosomal proteins have been recently associated with biotic stress responses [76], but their role in pathogenesis remains intriguing. Moreover, an uncharacterized secreted protein (Bcin15g00100) was expressed in all the samples analyzed in both studies, deserving special attention since the orthologous in Blumeria graminis was associated with virulence [77].

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with phosphate buffer. Samples were shortly placed on ice, frozen in liquid nitrogen, and kept at –80°C until further use. Preceding extraction for transcriptomics and metabolomics, seeds were eliminated and samples grinded in liquid nitrogen. Three-four biological replicates of Syrah and Trincadeira green berries (control and infected) were considered for RNA-sequencing and metabolomics.

**RNA extraction, sequencing, and gene expression analysis**

RNA was extracted as mentioned in Fortes et al. [78] with modifications [16]. RNA quantity and integrity were evaluated as previously [16]. Sequencing was performed at the Centre for Genomic Regulation (Barcelona). TruSeq Stranded mRNA Sample Prep Kit v2 (ref. RS-122-2101/2) was used to prepare the libraries. Libraries were sequenced (50 nt paired-end) on the Illumina HiSeq 2500 using v4 chemistry. Twelve libraries were sequenced for Syrah and Trincadeira samples (6 control and 6 infected). Illumina raw read data was placed in the NCBI Sequence Read Archive (SRA; PRJNA61792). Raw reads generated were checked for general quality and presence of adapters or contaminants via FastQC analysis [79]. Quality trimming and filtering of raw reads were done with an in-house script with a threshold of 30 (quality score). Ten nucleotides at 5’-end were trimmed from each sequence of all libraries.

Grapevine (12Xv1, http://genomes.cribi.unipd.it/) and B. cinerea (strain B05.10) (ASM83294v1, http://fungi.ensembl.org) were the selected reference genomes. HISAT2 (v. 2.1.0) aligned processed reads to the combined references to identify the splice sites independently of the annotations [80]. The mapping parameters were as follows: --rgd 2.2 --rfq 2.2 --mpq 2.2 --rna-strandness RF. The software package SAMtools (v. 1.3.1) (http://samtools.sourceforge.net/) was used for processing of the mapped reads, such as removal of duplicates (rmdup). The HTSeq tool (version 0.9.11) counted the strand-specific read-pairs mapped to the exon regions annotated in the grapevine (12Xv1) and B. cinerea (ASM83294v1) genomes [81]. Differential gene expression analysis was performed using the Bioconductor package EdgeR (v. 3.24.2) [82]. Total read counts were first normalized by library size using the trimmed means of median (TMM) method [82]. Normalization of depth and gene length were done by transforming pair-read to fragments per kb per million counts (RPKM). For differential expression analysis a RPKM >10 threshold was used; dispersion among samples was evaluated, and an ANOVA-like test was conducted for any pairwise comparison with exactTest function.

Differentially expressed grapevine genes (DEGs) were significantly changed at the FDR < 0.05 and log2 fold change (log2FC) > 1.0. B. cinerea genes were handled as present or absent, comparing infected vs. control samples for both cultivars. Genes were only considered present if normalized gene count was equal to zero in all three control replicates and higher than twenty in all three infected replicates. Moreover, B. cinerea genes present in Syrah and absent in Trincadeira were described as Syrah-specific, and vice versa.

**Functional enrichment analysis**

Significant functional enrichment in V. vinifera DEGs was identified with FatGO [35] and by using a grapevine-specific functional classification of 12X V1 genome assembly predicted genes [83]. Fisher’s exact test was performed to compare these outputs with the list of total non-redundant genes in the grapevine genome. Enrichment was significant for P-value <0.05 following Benjamini and Hochberg correction for multiple testing. Transcripts of B. cinerea were functional annotated according to [5] which was manually updated by literature review.

**Soluble metabolites**

Soluble metabolite profiling was performed by using Gas chromatography coupled to electron impact ionization time-of-flight mass spectrometry (GC-EI/TOF-MS) [84]. Soluble metabolites were extracted from deep-frozen powder in ethyl acetate for 2 h agitation at 30°C [85]. After centrifugation, two aliquots from the ethyl acetate fraction were dried by vacuum concentration and kept at –20°C.

Chemical derivatization and retention index calibration were conducted before injection [84]. GC-EI/TOF-MS analysis was performed using an Agilent 6890 N24 gas chromatograph (Agilent Technologies, Germany) connected to a Pegasus III time-of-flight mass spectrometer (LECO Instrument GmbH, Germany) [28]. Chromatograms were processed as previously detailed [28].

Identification of compounds was performed based on mass spectra and retention time index matching to Golm Metabolome Database [86, 87] and by using TagFinder software [88] (considering the presence of at least three specific mass fragments per compound and a retention index deviation of less than 1.0% [89]). Metabolite intensities were normalized with fresh weight and internal standard (C22), maximum scaled, and log-transformed to approximate normal distribution. A subset of metabolites was identified only by mass spectral match as indicated by square brackets, e.g. [ursolic acid]. This compound differs in retention index from expected [90] and identified abundant oleanolic acid (Supplemental Table S1) and matches best to ursolic acid. Due to a deviation from the expected retention index and the presence of other pentacyclic triterpenoids [91] in V. vinifera, we annotated [ursolic acid] as a triterpenoid of the ursolic/ oleanolic acid family.

**Volatile metabolites**

Volatile profiling (500± 50 mg fresh weight) was performed by solid-phase micro-extraction (SPME) and GC coupled to an electron impact ionization/quadrupole MS (GC-EI/QUAD-MS) using an Agilent 5975B VL GC–MSD system and according to Vallarino et al. [92]. SPME samples were removed from the headspace and processed as previously [28]. Programming for GC was 2 min isothermal at 40°C followed by a 10°C/min ramp to 260°C final temperature (held constant for 10 min). The Agilent 5975B VL GC–MSD system was operated with a continuous flow of helium at 1.0 mL/min. Desorption from the SPME fiber was at 16.6 psi with an initial 0.1 min pulsed-pressure at 25 psi. The subsequent purge was 1 min at a purge flow of 12.4 mL/min. System stability was controlled, and the sample sequence randomized. GC-EI/QUAD-MS chromatograms were acquired with the mass range set to 30–300 m/z and a 20 Hz scan rate. Chromatograms were obtained, visually controlled, and exported in NetCDF file format using Agilent ChemStation-Software (Agilent) and baseline-corrected with MetAlign software [93].

Compounds were identified matching to the reference collection of volatile compounds [86, 87] (presence of at least three specific mass fragments per compound and a retention time deviation of less than 3%). Metabolite intensities were then normalized by sample fresh weight, maximum scaled, and log-transformed to approximate normal distribution.

**Statistical analysis**

Metabolomics data (log2-transformed response ratios) was analyzed using Student’s t-test, one- and two-way ANOVA, Kruskal-
walls, and Wilcoxon rank-sum tests. Benjamini & Hochberg correction was used for multiple comparisons, MetaGeneAlyse web application was used for principal component analysis (v.1.7.1; http://metagenealyse.mpimp-golm.mpg.de) and the R function pnorm to the log2-transformed response ratios with missing value substitution, log2 = 0. Heatmaps were obtained using the R package ComplexHeatmap [94]. Venny 2.1 web tool (https://bioinfogp.cnb.csic.es/tools/venny/) was used for Venn diagrams.

Acknowledgments

Fundação para a Ciência e Tecnologia (FCT) supported the research through “GranelInfecções” (PTDC/ASP-HOR/28485/2017). This article/publication is based upon work from COST Action CA 17111 INTEGRAPE, supported by COST (European Cooperation in Science and Technology). FS and D.P. were recipients of fellowships from BioSys PhD programme PD65-2012 (PD/BD/114385/2016 and PB/Bd/130976/2017, respectively).

Author Contributions

A.M.F designed and supervised the study; F.S., D.P., A.M.F., extracted RNA; D.P., A.E., J.K., performed metabolomics; F.R., C.R., performed infections; F.S., M.P., M.G.C. performed bioinformatic analysis of RNAseq. C.N. Performed one figure and assisted in tables and final draft; F.S. wrote the initial draft, completed by A.M.F.; M.G.C.; J.K.; A.M.F revised the manuscript.

Data availability

RNA-seq data is available at the NCBI SRA database under PRJNA611792 accession number.

Conflict of interests

The authors declare no conflict of interest.

Supplementary data

Supplementary data is available at Horticulture Research online.

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