Grapevine Inflorescence and *Botrytis cinerea*: An Attempt to Understand the Molecular Queues during Initial Infection and Entrance into Quiescence

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Abstract

Botrytis cinerea, *a cosmopolitan nectrotrophic fungus, is the cause of gray mold in a wide range of crops including grapevine, where it affects both fruit quality and yield. The pathogen is difficult to manage due to the quiescent state of infection ensuing after the primary infection. Primary infection mostly occurs at bloom by airborne conidia; and the fungus remains quiescent until maturity and egresses at ripe to causes bunch rot. To understand the molecular crosstalk between the grapevine and* **B. cinerea** *during initial infection and entrance into quiescence, inflorescences of* **Vitis vinifera** *(cv. Pinot Noir) were inoculated with* **B. cinerea** *at anthesis. Infections were halted at 12, 24, 48, 72 and 96 hours post inoculation (hpi) and samples were subjected to confocal microscopy, quantitative polymerase chain reaction and RNA-sequence analyses. It was observed that most of the conidia germinated and form apressoria-like structures within 24 hpi, which resulted in penetration of the flower epidermis. During penetration,* **B. cinerea** *increased the expression of its genes encoding for virulence factors that instigated defense responses from the flowers side. The defense responses involved genes associated with the accumulation of pathogenesis-related proteins, stilbenoids, reactive oxygen species and cell wall reinforcement. At 96 hpi the transcriptional reaction appeared largely diminished both in the host and in the pathogen, implying* **B. cinerea** *became quiescent, forced by the defense responses of the host, until conditions favor egression.*

Keywords: *Botrytis cinerea*; defense response; grapevine; inflorescence; quiescence.

Introduction

Grape vines (*Vitis* spp.) are among the most worldwide-grown perennial fruit crops with a global production of more than 80 million tons (FAOSTAT, 2017). Major economic benefits come from wine and related fermented products, but also from fresh fruit, raisins, fruit juices and jams. However, its production is jeopardized by adverse environmental conditions and biotic threats. The crop is prone to several diseases and pests, and their management consumes a large

proportion of its cultivation cost (Creasy and Creasy, 2009). Diseases from phytopathogenic fungi and oomycetes like *Plasmopara viticola*, *Erysiphe necator*, and *Botrytis cinerea* are the major cause of damage in quality and yield of grape.

Botrytis cinerea, a cosmopolitan necrotrophic fungus, causes pre- and post-harvest diseases in a wide range of crops including grapevine. The fungus can live pathogenically but also saprophytically or endophytically (van Kan et al., 2014; Shaw *et al*., 2016). Besides the different mode of infections the pathogen has, it propagates by mycelia and/or conidia, and survives for extended periods as sclerotia (Williamson *et al*., 2007). During infection, *B. cinerea* induces necrosis by producing toxins and reactive oxygen species (ROS) (van Kan, 2006), and manipulates host metabolism to facilitate colonization (Williamson *et al*., 2007). *Botrytis* can quiescently infect immature fruits where that damage is mostly exhibited during ripening. Host factors that derive the pathogen into quiescent are proposed to be the presence of preformed and inducible antifungal compounds, inactivation of fungal pathogenicity factors, and firm berry skin (Prusky *et al*., 2013). Thickness and permeability of grape berry cuticle and its epicuticular waxes, the biochemical composition and morphology of the berry skin, and ripening stage are described as important berry skin features regarding the resistance/susceptibility of berries to *B. cinerea* (Deytieux-Belleau *et al*., 2009; Becker and Knoche, 2012; Herzog *et al*., 2015).

In vineyards, *B. cinerea* is part of the natural microflora where previous-season infection is the main source of primary infection (Nair *et al*., 1995). *Botrytis* infection is possible to occur at any stage of berry development, but primary infection often occurs during bloom (McClellan and Hewitt, 1973; Nair *et al*., 1995; Keller *et al*., 2003). A mechanistic model based study suggested that infection status on inflorescences and young clusters explain well *B. cinerea* epidemics in vineyards (González-Domínguez *et al*., 2015). Higher disease severity, at harvest, was observed from grapevine inflorescences received *Botrytis* inoculation at full bloom, (Keller *et al*., 2003). After the initial infection at bloom, the pathogen generally remains quiescent until the onset of fruit ripening and it resumes active growth thereafter to cause bunch rot (McClellan and Hewitt 1973; Keller *et al*., 2003). Thus, bunch rot observed at maturity may not only be due to *de novo* infection of mature berries, it rather could be from the latent infection established earlier at bloom.

Studies so far conducted on the quiescent infections of *B. cinerea* in grapevine inflorescences (for example: McClellan and Hewitt, 1973; Nair *et al*., 1995; Keller *et al*., 2003) demonstrated that flower infection is an important stage in the epidemiology of *B. cinerea* in grapevine. The intriguing phenomena that forces *B. cinerea* stay quiescent until grape

berry ripening remained are not fully understood. Though reprogramming of carbohydrate and lipid metabolisms towards increased synthesis of secondary metabolites involved in plant defense, such as resveratrol phytolexin, was observed during incompatible interaction of *B. cinerea* and unripe grape berries (Agudelo-Romero *et al*., 2015; Kelloniemi et al., 2015). Thus, the crosstalk between *B. cinerea* and grapevine during initial infection at bloom remains as a subject for research, and therefore this study aimed at understanding the molecular queues entailed at initial infection and entrance to quiescence.

Materials and Methods

Plant material and *B. cinerea* inoculation

Fruiting cuttings of cv. Pinot Noir, collected from an experimental vineyard of the Fondazione Edmund Mach, were used to raise inflorescences. Cuttings with 3-4 nodes were induced to root by treating with 300 ppm of indol-3-butyric acid for 15 min. After five to six weeks, individual rooted cuttings were repotted into a 1.5 l pot using commercial potting mix. Following budburst, the vegetative apex was removed to promote inflorescence growth. *B. cinerea* (isolate B05.10) was incubated on potato dextrose agar (PDA) on plate at 25 °C. Conidia were harvested with distilled water from 10 days old plate, and concentration was adjusted to $2 * 10^5$ conidia ml^{-1} using hemacytometer. At full cap-fall stage, each flower received 1.5 µl of a conidial suspension or distilled water (mock inoculation) closer to receptacle area. Inoculated cuttings were immediately bagged by water sprayed, clear plastic bag for 24 h to create high humidity. A transformant strain of B05.10, expressing green fluorescent protein (GFP) was used for microscopic observation and for post-inoculation evaluation of flowers/fruitlets.

Microscopic observation and detection of quiescent *B. cinerea*

Thin slices of fertilized gynoecia were cut for confocal microscope observation about the state of *B. cinerea*. While for detecting the presence of quiescent *B. cinerea* in the first 14 days post inoculation, 8 fruitlets from each of six biological replicates were plated out on PDA (with Hygromycin B at 70 µg ml^{-1}), before or after washing, for a week at room temperature. Washing was made with sterile water, a 1 minute three rinses with gentle shaking. An inflorescence from a fruiting cutting was considered as a biological replicate.

RNA extraction, quantitative polymerase chain reaction (qPCR) and RNA-sequence analyses

Samples were collected at 12, 24, 48, 72 and 96 hpi in three biological replicates and snap frozen in liquid nitrogen, and kept at -80 °C until use.

RNA was extracted using Plant Total RNA Kit following the manufacturer's protocol. For qPCR assays, cDNA was synthesized from 3 µg of RNA, using SuperScript™VILO™cDNA Synthesis Kit. qPCR was performed in a Viia7 thermocycler using 0.31 µl of cDNA and 2.5 µM of primers in a total volume of 12.5 µl, where half of the total volume was Fast SYBR Green Master Mix. The reference genes used were *VvActin* and *VvTub*, and *Bcrpl5* and *BcTubA*, for grapevine and *B. cinerea*, respectively. All primers and corresponding gene identifiers are provided in Table 1. Expression level of a gene was determined relative to its expression level in mock inoculated samples or PDB cultured *B. cinerea*. Statistical significance was calculated by Tukey's Honestly Significant Difference test or unpaired heteroscedastic Student's *t* test.

Table 1. Genes name and primers sequence used in quantitative polymerase chain reaction

Gene name	Forward and Reverse primer sequences
BcACTA	CGTCACTACCTTCAACTCCATC; CGGAGATACCTGGGTACATAGT
BcBOA6	CAGCAATCGTTGTCCTGAAATC; GTTTATCGCGTTCTTCACCTGTTA
BcCUTA	TGCTGGCAGTCAGACTATGG; TTCGGCTGGTAAAAGTTTGG
BcTUBA	TTTGGAGCCAGGTACCATGG; GTCGGGACGGAAGAGTTGAC
BcβGLUC	TGCAGCTACCTTTGATCGTG; TCCTTCCCAGTTACGTCCAC
Vv4CL	TTCCCGACATCAACATCCCG; TTACGTGCGGTGAGATGGAC
VvACT	ATGTGCCTGCCATGTATGTTGCC; AGCTGCTCTTTGCAGTTTCCAGC
VvCAD	GTGGAGGTGGGATCAGATGT; TCCATCTCTGATTTGCATGG
VvCCR	AGCAGAAACAGGGATGCCAT; AGAGAGCCTCCCATCTGACA
VvMYB14	TCTGAGGCCGGATATCAAAC; GGGACGCATCAAGAGAGTGT
VvPR10.1	GCACATCCCGATGCCTATTAAG; ACTTACTGAGACTGATAGATGCAATGAATA
VvPR10.3	GAAATCCTACAAGGACAGGGAGGT; CGGCCTTGGTGTGGTACTTTT
VvST29	GGTTTTGGACCAGGCTTGACT; GAGATAAATACCTTACTCCTATTCAAC
VvST41	GAGTACTATTTGGTTTTGGACCT; AACTCCTATTTGATACAAAACAACGT
VvTUB	TGTTGGTGAAGGCATGGAGG; AGATGACACGCCTGCTGAACT
VvWRKY33	ATTCAAGCACTAGTATGAACAGAGCAG; CCTTGTTGCCTTGGCATGA

Samples from 24 and 96 hpi were subjected for RNA-seq analysis a Next Generation Sequencing Platform HiSeq 1500 (Illumina, San Diego, CA). Genome assemblies of grapevine (Grape genome 12Xv1, http://genomes.cribi.unipd.it/) and *B.cinerea* (strain B05.10) (EnsemleFungi, http://fungi.ensembl.org) were used as reference sequences. The alignment was made by Subread aligner and raw read counts were extracted using the feature Count read summarization program. Genes were considered differentially expressed if they had fold change of ≥ 1.5 and *p*-value < 0.05. Gene ontology enrichment was computed using customized annotation and annotated reference of GO terms into the AgriGO analysis tool (http://bioinfo.cau.edu.cn/agriGO/analysis.php; Du Z *et al.* 2010) and enriched GO terms (FDR <0.05) were visualized using the 'Reduce + Visualize Gene Ontology' (REViGO) webserver (http://revigo.irb.hr; Supek *et al*., 2011).

Results and Discussion

Botrytis cinerea infection of grapevine inflorescence

Visualization of inoculated flowers at 24 hpi (Figure 1A) with confocal microscopy revealed that within 24 hours fungal conidia germinate, develop appressoria, and penetrate into the flower cuticle, on gynoecium above the floral disc (Figure 1B). Despite this, there was no subsequent visible progress in the infection process and fruitlets look macroscopically healthy (Figure 1C-D). However, the plating out activity carried out on these healthy looking fruitlets showed that about 90% of them, either washed or not, carried *B. cinerea* that they received at cap-off stage (Figure 1E). The presence of the pathogen without visible symptom or sign for such a longer period may suggest that the fungus was not actively growing to cause disease, entered into quiescent phase after penetrating the flowers cuticle.

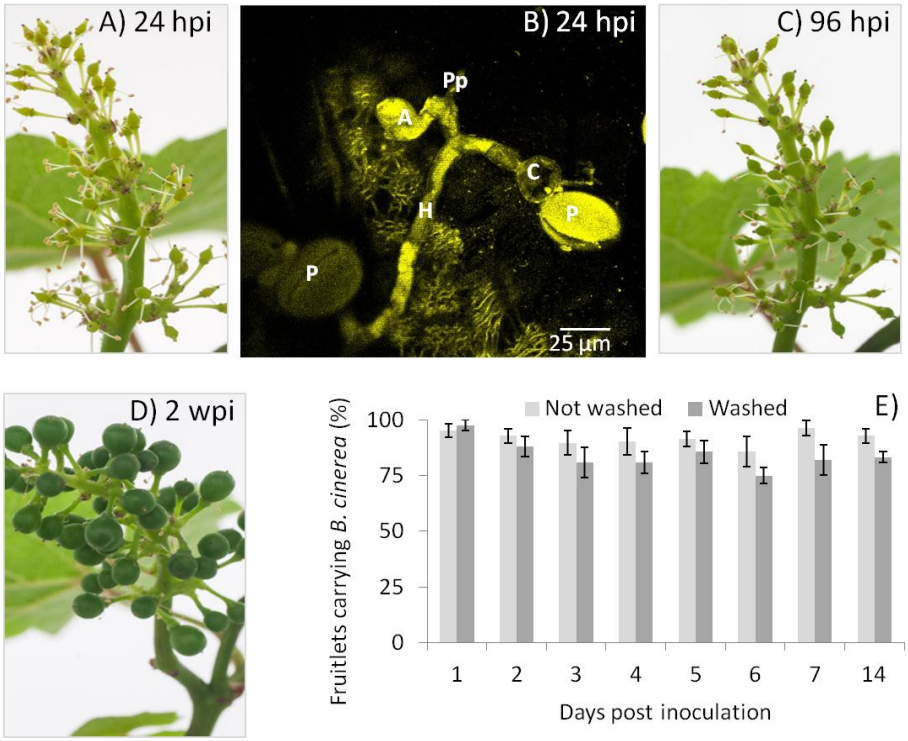

Figure 1. *Botrytis cinerea* infected grapevine flowers. Flowers inoculated with GFP-labelled B05.10 at full cap-fall stage (**A**) at 24 hours post inoculation (hpi), (**B**) confocal microscope image showing the fungus infecting flower cuticle at 24 hpi (**C**) asymptomatic fruitlets at 96 hpi and (**D**) 2 weeks post inoculation (WPI). *A, appressoria*; *C, conidium*; *H, hypha*; *P, pollen* grain; Pp, penetration peg. (**E**) Plating out of infected fruitlets on selective media (PDA with Hygromycin B, 70 µg/ml) before (NW) or after washing (W). Values at each day represent mean proportion of fruitlets showing GFP-labelled B05.10 growth on the selective media. Error bars indicate standard error. One way ANOVA computed on square root transformed data showed that mean proportions of W and NW, of each day, was not significantly different ($P \geq 0.173$). Also mean proportions throughout the two weeks within W or NW was not significantly different ($P \geq 0.160$).

Sequencing the infected flowers transcriptomes

The biological variability within replication and among experimental conditions was assessed using hierarchical clustering, based on Spearman's correlation. The results indicated that there is a good degree of correlation within biological replicates of each experimental condition at 24 than 96 hpi (Figure 2A). From the hierarchical clustering, two distinct clusters were formed by 24 and 96 hpi, irrespective of treatment conditions, except for "Ctrl 3-96" and "Trt 1-24" (Figure 2A). The latter one has higher correlation value to the other two biological replicates of its kind than the rest of all. In the first cluster, which was comprised by samples from 24 hpi, the two inoculated samples were closer to each other than mock inoculated ones. However, in the second cluster, comprised by samples from 96 hpi, there were no such distinct differences among *B. cinerea* and mock inoculated samples, suggesting that samples at 96 hpi were similar at a whole transcriptome level.

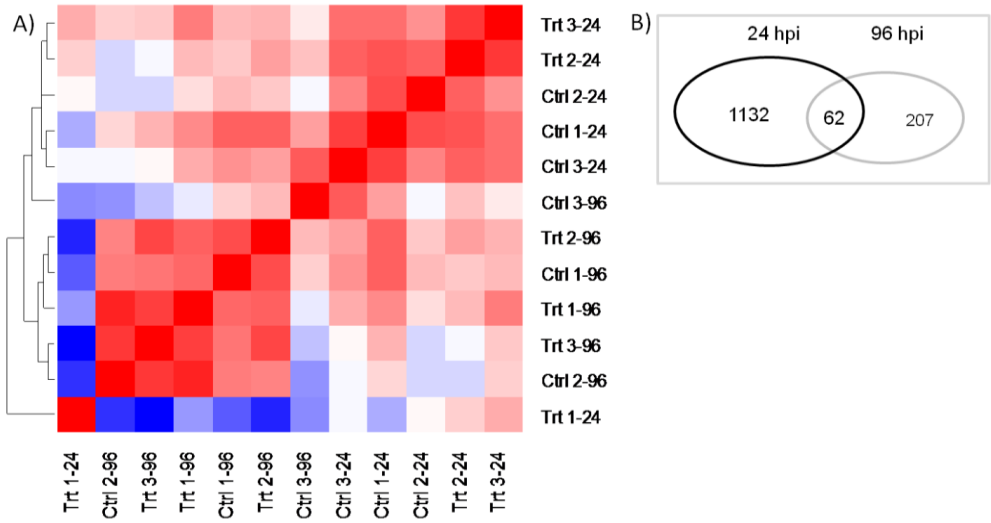

Figure 2. Global evaluation of the RNA-seq experiment and total differentially expressed (DE) genes. (**A)** Heat map of Spearman's correlation of the 12 samples. Ctrl, mock inoculated; Trt, *B. cinerea* inoculated; Bc, *Botrytis cinerea;* 1-3 indicates the biological replicates; and 24 and 96 are hours post inoculation (hpi). (**B**) Numbers of differentially expressed genes ($P < 0.05$, fold change > 1.5) up on *Botrytis cinerea* infection at 24 and 96 hpi. Differential expression of grapevine genes was computed from the total expressed genes (20,530 out of the total 29,970 *V. vinifera* genes) in the inflorescences. Of these expressed genes, 1401 of them were differentially expressed (DE): 1194 DE genes at 24 hpi, whereas only 265 DE genes at 96 hpi (Figure 2B). The common DE genes between the two time points was limited to 62. Interestingly, at 24 hpi, the plant seems to respond to the presence of the pathogen with a prevalent induction of genes, which appeared to be no longer modulated at 96 hpi.

Botrytis cinerea transcripts detected in planta during grape flower infection

According to the RNA-seq analysis, 836 *Botrytis* genes had raw reads of more than 10, and this set of genes was referred as *in planta* detected genes (Figure 3A). Of these 836 genes, 818 and 287 were detected at 24 and 96 hpi, respectively; 269 genes being common both for 24 and 96 hpi (Figure 3B). GO slim analyses were performed on the 836 *in planta* detected genes. Among the most represented GO terms in biological processes were related to translation, carbohydrate metabolism, energy metabolism, and anabolic and catabolic processes (Figure 3B), which are suggestive for cell wall degradation being activated and thereby initiation of infection. In the case of molecular functions ontology, functions related to ion binding and oxidoreductase activity were represented highly, indicating a widespread reprogramming of fungal transcriptome for pathogenesis. For example, oxidoreductase activity, a functional class represented most at 24 hpi than 96 hpi (Figure 3B), comprises genes involved in ROS production and scavenging activities, which is an important *B. cineria*'s pathogenesis mechanism. Overall, the molecular functions associated to the *in planta* detected *Botrytis* genes were consistent with the functional categories in biological processes. In the case of cellular components ontology, since there were a lot of translational activities, as expected, "Ribosome" and "Protein complex" were among the most represented ones (Figure 3B). Furthermore, overrepresentation analysis of the *in planta* detected genes, based on annotated *B. cinerea* genes (Amselam *et al*., 2011; Blanco-Ulate *et al*., 2014; Espino *et al*., 2010; Schumacher *et al*., 2014), showed that genes encoding for proteins of early secretome, enzymes acting on plant cell wall (mainly on hemicellulose and pectin), and generation and deactivation of ROS were overrepresented (Table 2).

As depicted in Figure 4, genes involve in *Botrytis* virulence were upregulated more at 24 than 96 hpi. The higher expression of *B. cinerea* cutinase (*BcCUTA*) at 24 hpi implies the breaching of cuticle layer by appressoria. Similar expression profile was also observed by another cell wall degrading enzyme beta-glucosidase (*BcβGLUC*), degrading both cellulose and hemicelluloses. Botcinic acid (*BcBOA6*) gene encodes a polyketide synthase, one of the key enzymes in the biosynthesis of phytotoxic secondary metabolites.

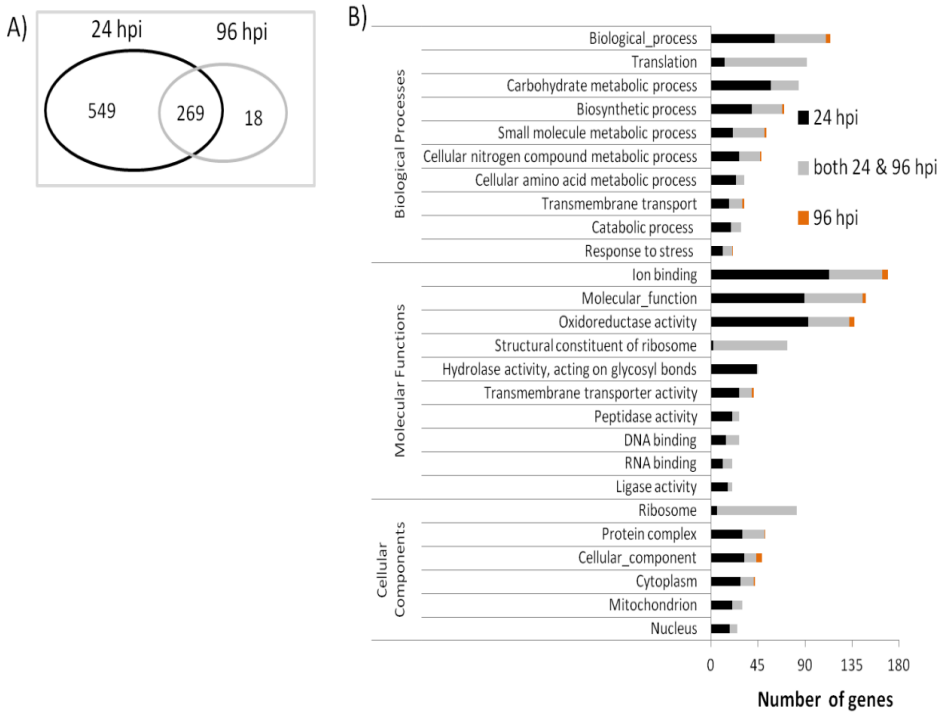

Figure 3. Number of detected *Botrytis cinerea* genes and their associated GO terms. (**A)** Venn diagram showing the number of detected genes unique or common to 24 and 96 hpi. (**B)** GO slim terms of *B. cinerea* genes detected *in planta*.

For *B. cinerea*, breaching plant cell wall is a key process to establish infection (van Kan, 2006; Williamson *et al*., 2007; Blanco-Ulate *et al*., 2014). After penetrating the cuticle, when infection is successful, the fungus grows into a pectin-rich epidermal cell (van Kan, 2006; Williamson *et al*., 2007) by deploying its pectinases. Within 24 hpi, genes encoding for enzymes involve in cell wall depolymerisation were observed enriched and expressed significantly (Table 2 and Figure 4). Despite the observed readiness of the fungus to infect the grapevine flowers, there was no visible *Botrytis* symptom observed though the presence of quiescent *B. cinerea* was confirmed (Figure 1E). This implies that, the pathogen could not able to grow actively due to the fierce defense it faced from the flowers. According to Prusky (2013), quiescence of a pathogen can happen at different developmental stages like before or after conidia germination / appressoria formation, and/or at subcuticular hyphae stage. Quiescent infections can occur in cellular locations like cuticular wax, intercellular space, and epidermal cells (Prusky *et al*., 2013). In unripe tomato, *Colletotrichum gloeosporioides* was reported to enter into quiescent as a swollen hayphae after its appressorium germinated in to the cuticular wax (Alkan *et al*., 2015). With regard to *B. cinerea* in grapevine, hyphae in the epidermal cell of immature berries were supposed to stay quiescence (Keller *et al*., 2003).

Table 2. Functional categories of *Botrytis cinerea* genes detected in the inoculated grapevine flowers

Functions of selected *Botrytis cinerea* genes	Total no. of genes	24hpi			96hpi		
		no. of detected genes	pvalue	odds ratio	no. of detected genes	pvalue	odds ratio
Total number of predicted genes (B05.10 strains) (Amselem et al 2011)	16448	818			287		
Proteins identified as early secretome, within 16 h of germination (Espino et al., 2010)	102	34	0.00E+00	6.7	7	2.30E-02	3.93
Carbohydrate-Active Enzymes (CAZymes) (Blanco-Ulate et al 2014)	1155	150	0.00E+00	2.6	28	8.50E-02	1.39
CAZymes acting on fungal cell wall	166	26	0.00E+00	3.1	4	2.90E-01	1.38
CAZymes acting on Plant Cell Wall	151	51	0.00E+00	6.8	5	1.44E-01	1.90
CAZymes acting on cellulose	10	3	7.50E-02	6.0	0	9.99E-01	0.00
CAZymes acting on hemicellulose	58	18	0.00E+00	6.2	2	2.43E-01	1.98
CAZymes acting on hemicellulose and pectin side chains	28	7	0.017	5.0	0	1.00E+00	0.00
CAZymes acting on pectin	55	18	0.00E+00	6.6	1	4.84E-01	1.04
Proteins generating Reactive Oxygen Species (ROS) (Schumacher et al., 2014)	53	8	2.90E-02	3.0	1	4.70E-01	1.08
Proteins involved in the detoxification of ROS (Schumacher et al., 2014)	98	20	0.00E+00	4.1	5	6.90E-02	2.92
Protease (Amselem et al 2011)	377	30	2.00E-02	1.6	5	7.59E-01	0.76
Secondary metabolism key enzymes (Amselem et al 2011)	42	2	0.524	1.0	0	1.00E+00	0.00
60S & 40S ribosomal protein (Amselem et al 2011)	81	77	0.00E+00	19.1	77	0.00E+00	54.48
Appressorium-associated genes (orthologs in *Magnaporthe oryzae)* (Amselem et al 2011)	12	6	1.40E-02	10.1	2	1.02E-01	9.55
Transporters (Amselem et al 2011)	442	52	0.00E+00	2.4	17	1.00E-02	2.20
Transcription factors (Amselem et al 2011)	410	19	6.23E-01	0.9	4	9.41E-01	0.56

Significance was calculated using Fisher's Exact test. P values (<0.05) and odds ratios higher than 2 are highlighted

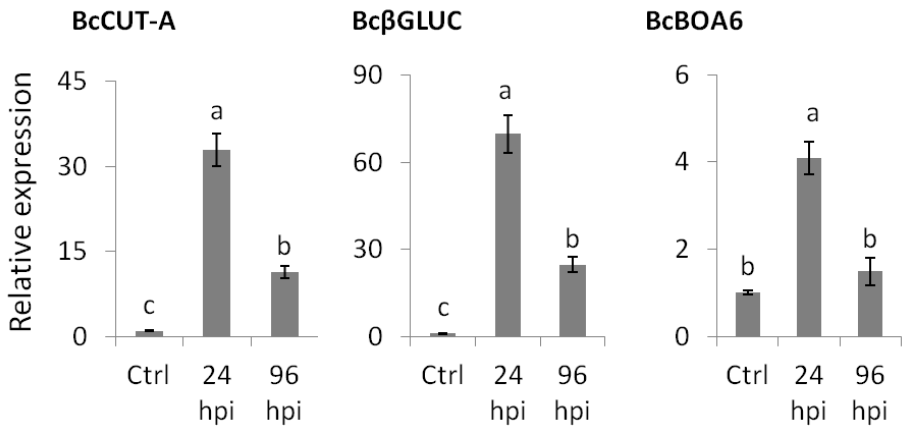

Figure 4. Expression profile of virulence-related *Botrytis cinerea* genes during grapevine flower infection [at 24 and 96 hours post inoculation (hpi)] relative to PDB cultured *B. cinerea*. BcCUTA (*B. cinerea* cutinase A), BcβGLUC (*B. cinrea* beta-glucosidase), BcBOA (*B. cinerea* botcinic acid), and ctrl (PDB cultured *B. cinerea*). Error bar is the standard error of the mean of three biological replicates. Expression values followed by a common letter are significantly not different among ctrl, 24, and 96 hpi samples, according to Tukey's Honestly Significant Difference test ($P \leq 0.05$), using one way ANOVA.

From the microscopic and plating out activities of this study, *B. cinerea* entered into quiescent state after penetrating the first few epidermal cell layers, although quiescence as ungerminated conidia could also be possible.

All together, from the microscopic and transcriptomic results, the fungus attempted to infect grapevine flowers before entering into quiescence. Appressoria germination into flower gynoecium (Figure 1B), enrichment of functional categories required for causing disease such as plant cell wall degrading enzymes and ROS (Table 2) and differential regulation of virulence-related genes (Figure 4) were indications for the readiness of the pathogen to establish infection. In addition to these facts, most of the *in planta* detected *Botrytis* genes were also found highly expressed during successful infection of lettuce (De Cremer *et al*., 2013), tomato (Smith *et al*., 2014), and ripe grapevine berry (Kelloniemi *et al*., 2015).

Transcriptional alterations of grapevine inflorescence upon *B. cinerea* infection

Gene ontology (GO) term enrichment analyses were made to evaluate the biological processes, molecular functions, and cellular components affected most due to *B. cinerea* infection (Table 3). At 24 hpi, significantly enriched (FDR < 0.05) biological processes include many defense responses, for example, responses to biotic stimulus, biosynthesis of secondary metabolites, and protein modification process. In accordance to this defense related activities, signal transduction and kinase activities, carbohydrate binding, and transferase activity were also significantly overrepresented GO terms of molecular functions. From the

RNA-seq analysis, all sorts of genes that take part during biotic stress were differentially regulated. In the signaling pathway, among others, wall-associated kinases and phytohormones were observed to involve at the initial stage of host-pathogen interaction (Table 4). Different genes of cell wall and secondary metabolism were also induced.

Table 3. Gene ontology terms enriched in the differentially expressed grapevine genes upon *B. cinerea* infection. Enriched GO terms at 24 and 96 hours post inoculation are presented.

HPI	GO term	Ontology	Description	FDR
24	GO:0019748	BP	secondary metabolic process	2.60E-32
24	GO:0009607	BP	response to biotic stimulus	6.90E-08
24	GO:0009875	BP	pollen-pistil interaction	1.70E-04
24	GO:0006464	BP	protein modification process	8.70E-05
24	GO:0007049	BP	cell cycle	1.30E-04
24	GO:0009056	BP	catabolic process	8.80E-04
24	GO:0003774	MF	motor activity	4.40E-09
24	GO:0030246	MF	carbohydrate binding	1.50E-04
24	GO:0016301	MF	kinase activity	9.50E-03
24	GO:0004871	MF	signal transducer activity	5.80E-03
24	GO:0016740	MF	transferase activity	6.80E-03
24	GO:0005576	CC	extracellular region	5.60E-04
96	GO:0005576	CC	extracellular region	1.80E-06
96	GO:0005618	CC	cell wall	8.10E-05

NB: BP, biological process; MF, molecular function; CC, cellular component.

Following inoculation, the infection attempt of *Botrytis* instigated arrays of responses from the grapevine inflorescence. As shown in Table 3 and 4, signaling and receptors were differentially expressed within 24 hpi, which are known to involve in immune response to pathogens. Over-expressing *WAK1* enhanced resistance to *B. cinerea* in *Arabidopsis* (Brutus *et al*., 2010). Conversely, *BAK1* mutant of *Arabidopsis* induced susceptibility to *Sclerotinia sclerotiorum* and *B. cinerea* (Kemmerling *et al*., 2007). The expression of *BAK1* and *WALK1* membrane receptors increased in *V. pseudoreticulata* in response to *E. necator* (Weng *et al*., 2014) and in lettuce due to *B. cinerea* (De Cremer *et al*. 2013). The upregulation of these genes in this study indicated that the plant recognized *Botrytis* intrusion to put defense responses in line. Consequently, quick and strong induction of PR proteins and accumulation of stress related secondary metabolites, as well as cell wall fortification were deployed as major defense responses to halt the infection.

Genes encoding prominent transcription factors (TF), such as WRKY and MYB TFs, known to involve in plant-pathogen interaction, were also differentially expressed. From the qPCR assay, the transcriptional profile of *VvWRKY33* was higher at 12 and 24 hpi (as compared to mock-treated samples)

and dropped to the control level at later time points, 48 hpi and beyond, while the pathogenesis-related (PR) proteins were always higher than control, except *VvPR10.1* at 48 hpi (Figure 5A). A very quick induction of *VvPR10.1* and *VvPR10.3*, coinduced with *VvWRKY33*, suggested that these PR proteins involved in grapevine flower resistance against *B. cinerea*. Merz *et al*. (2015) reported that *VvPR10.1* involves in *P. viticola* resistance of grapevine under the regulation of *VvWRKY33*. *WRKY33* in *Arabidosis*, a functional homology of *VvWRKY33*, confer resistance to *B. cinerea* by regulating redox homeostasis, phytohormonal signaling, and biosynthesis of phytoalexin (Birkenbihl *et al*., 2012). The other induced PRs (Table 4), such as chitinases and glucanase, both hydrolytic enzymes, contribute to grapevine resistance by degrading structural components in fungal cell walls (Giannakis *et al*., 1998). Thaumatin protein has strong anti-fungal activity in vitro by blocking the growth of mycelia of *Phomopsis viticola* and *B. cinerea* (Monteiro *et al*., 2003), implying to have role in resistance of grapevine against fungal pathogens. The other defense mechanism got activated was the secondary metabolite biosynthesis.

Table 4. Selected *Botrytis*-induced genes in grapevine flower at 24 and 96 hpi (with P-value of ≤ 0.05 and absolute fold change of ≥ 1.5)

Gene Id	Fold change (log2)		Functional annotation	Category
	24 hpi	96 hpi		
VIT_02s0234g00130	1.60		Ethylene responsive element binding factor 1	Recognition & signaling
VIT_11s0016g00710	0.83		Jasmonate ZIM-domain protein 1	
VIT_00s0258g00140	1.26		Brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1)	
VIT_17s0000g04400	1.38		Wall-associated kinase 1 (WAK1)	
VIT_14s0128g00970	2.75	1.40	Germin-like protein 3	Cell wall
VIT_06s0009g02560	3.21		Pectinesterase family	
VIT_06s0004g01990	4.87	3.15	Proline-rich extensin-like family protein	
VIT_11s0149g00280	2.13		Chitinase A	Response to stress & secondary metabolism
VIT_08s0007g06060	1.60		Beta 1-3 glucanase	
VIT_05s0094g00320	2.65		Chitinase, class IV	
VIT_07s0005g02560	2.32		Chitinase Class I	
VIT_02s0025g04230	2.20		Thaumatin	
VIT_03s0088g00890	1.42		Pathogenesis related protein 1 (PR1)	
VIT_01s0010g02020	7.12	2.09	Peroxidase	
VIT_16s0039g01280	5.40		Phenylalanin ammonia-lyase	

The other spectrum of defense mechanism activated following *Botrytis* inoculation was the secondary metabolism. Genes encoding stilbene biosynthesis, *VvSTS29* and *VvSTS41*, and their regulator VvMYB14 TF were differentially regulated (Figure 5B). Stilbenes have phytoalexin property (Favaron *et al*., 2009). Fortification of cell wall was also

hinted from the upregulation of genes involved in monolignol biosynthesis pathway, a precursor for lignin synthesis (Figure 5C). Genes encoding 4-coumarate-CoA ligase (*Vv4CL*), an enzyme in the upstream of the pathway, cinnamoyl CoA reductase (*VvCCR*), the first enzyme specific to monolignol synthesis, and Cinnamyl alcohol dehydrogenase (*VvCAD*), the final enzyme in the sequential reactions in the biosynthesis, were all differentially regulated at 12 and 24 hpi, *VvCAD* at 48 hpi as well (Figure 5C).Taken together, the grapevine fruitlets undertook a remarkable transcriptional reprogramming towards defense at the earlier time of *Botrytis* infection, i.e. within 24 hpi.

Genes encoding for the TF regulating stilbene biosynthesis, *VvMYB14* (Höll *et al*., 2013), and *VvSTS29* and *VvSTS41* were switched on following the infection. Studies have shown that stilbenes have phytoalexin nature and known to affect the growth of *B. cinerea* (Favaron *et al*., 2009). A transgenic *Arabidopsis*, which overexpresses a STS gene, increased its resistance to *Colletotrichum higginsianum* (Liu *et al*., 2011). In line with this, over-expressing the STS gene helped transgenic grapevine plants to resist *B. cinerea* colonization (Dabauza *et al*., 2014).

Reinforcing cell wall denies pathogens access to epidermal layer. The upregulation of monolignol genes were shown to help resistance in wheat against *Blumeria graminis* f. sp. *Tritici* (Bhuiyan *et al*., 2009). Whereas, silencing certain key genes of the monolignol biosynthesis pathway, such as PAL and CAD, compromised penetration resistance of wheat to the pathogen (Bhuiyan *et al*., 2009). In the grapevine fruitlets, the analyzed monolignol biosynthesis genes (Figure 5C), involved from upstream to downstream of the pathway, were differentially regulated following *B. cinerea* inoculation starting from as early as 12 hpi, suggesting cell wall apposition was in effect at such early time of interaction.

In conclusion, the microscopic and transcriptomic studies of this research provided good insight about the interaction between *B.cinerea* and grapevine inflorescence during the first 96 hpi. Within 24 hpi, *B. cinerea* genes required for establishing successful infection, such as those encoding cell wall degrading enzymes and phytotoxins, were induced. Wall associated receptor kinases of the inflorescences recognized the infection attempt and activated signal transduction, to reprogram inflorescences' transcriptome to "defense-oriented mode". As a result, enhanced expression of PR superfamily, stilbenoids (phytoalexins), and lignin biosynthesis (to stiffen cell wall) were put in place to tackle *B. cinerea* advancement. These defense responses were effective as at 96 hpi the numbers of *in planta* detected *B. cinerea* genes were evidently low, as compared to the initial infection stage; suggesting the

fungus switched to basal biological activity, i.e. entered to quiescent state. This was further confirmed by reduced number of differentially regulated defense related genes of the inflorescences at 96 hpi. The expression profiles of the defense responses of grapevine inflorescence that put *B. cinerea* into quiescent should be further studied to better understand their involvement in relation to ontogenic resistance. Molecular crosstalk of *B. cinerea* and grape berry at different growth stages and infection states should be further investigated for devising infection state/growth stage specific management of the fungus.

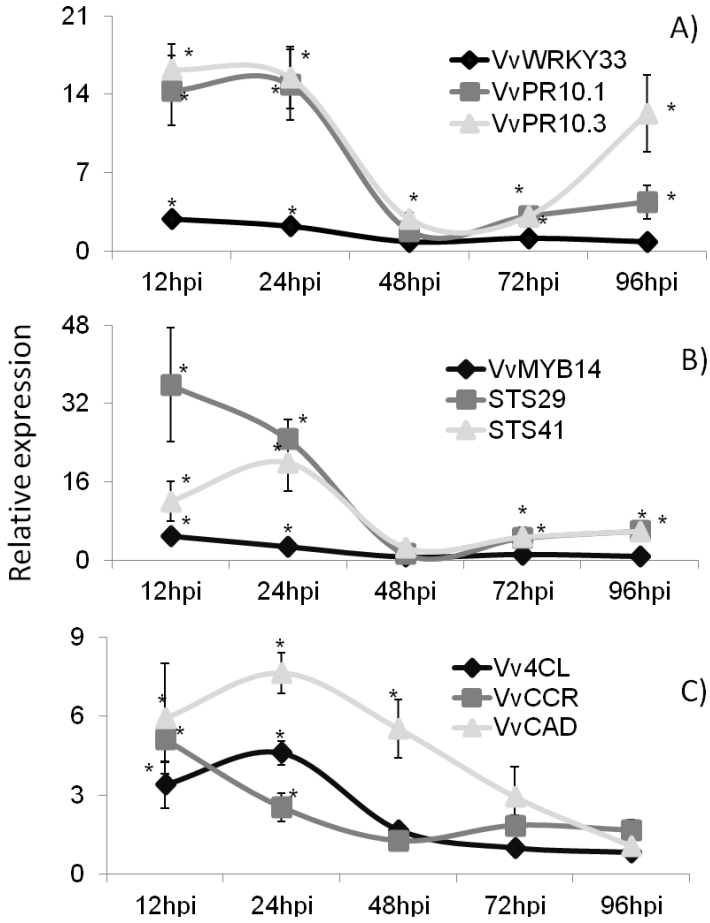

Figure 5. The expression profile of transcription factors and defense related genes [at 12, 24, 48, 72, and 96 hours post inoculation (hpi)]. (**A)** Expression profile of VvWRKY33 TF and coexpressed PR genes, VvPR10.1, and VvPR10.3. (**B)** Expression of Stilbene synthase genes VvSTS29 and VvSTS41, and their regulator Myb14 transcription factor. (**C)** Expression of critical enzymes in monolignol biosynthetic pathway, VvCCR (cinnamoyl CoA reductase), Vv4CL (4-coumarate-CoA ligase), and VvCAD (cinnamyl alcohol dehydrogenase). Error bar is the standard error of the mean of three biological replicates. Asterisks (*) indicate statistically significant difference ($P < 0.05$) between mock- and *Botrytis cinerea*- inoculated samples within post-inoculation hours using unpaired heteroscedastic Student's t test.

Acknowledgments

The authors would like to thank the Autonomous Province of Trento (PAT-ADP 2013–2016) for funding the research.

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