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# A MOLECULAR STUDY OF THE *Botrytis cinerea - Vitis vinifera* INTERACTION: FROM INFLORESCENCE TO RIPE BERRY

*presentata da*

Zeraye Mehari Haile

**Relatore:**

Dr. ELENA BARALDI

**Coordinatore:**

Prof. GIOVANNI DINELLI

**Correlatore:**

Dr. CLAUDIO MOSER

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**Declaration**

I, Zeraye Mehari Haile, hereby declare that this thesis is my own work with due acknowledgement of other materials used. I further state that the thesis has not been submitted for any award at any other university than the Univeristy of Bologna.

Zeraye Mehari Haile

# Contents

# List of Abbreviations

| | |
|---|---|
| % | Per cent |
| < | Less than |
| > | Greater than |
| °C | Degree Celsius |
| µm | Micrometer |
| 4CL | 4-coumarate-CoA ligase |
| ABA | Abscisic acid |
| ABC | ATP Binding Cassette |
| ACT | Actin |
| ANOVA | Analysis of variance |
| AOX | Alcohol oxidase |
| AP | Appressoria |
| AP8 | Aspartic proteinase |
| BAK | Brassinosteroid insensitive 1– associated kinase |
| Bc | *Botrytis cinerea* |
| BOA6 | Botcinic acid |
| BOT | Botrydial biosynthesis |
| BR | Brassinosteroid |
| C4H | Cinnamate 4-hydroxylase |
| Ca | Calcium |
| CAD | cinnamyl alcohol dehydrogenase |
| CCoAMT | Caffeoyl-CoA O-methyltransferase |
| CCR | Cinnamoyl CoA reductase |
| cDNA | Complementary DNA |
| CHS | Chalcone synthase |
| Co | Conidium |
| COMT | caffeic acid o-methyltransferase |
| Ct | Cycle threshold |
| Ctrl | Mock inoculated (Control) |
| CUTA | Cutinase |
| CUT-like1 | Cutinase |
| CWA | Cell wall apposition |
| CWDE | Cell wall degrading enzyme |
| DE | Differentially expressed |
| DFR | Dihydroflavonol-4-reductase |
| DNA | Deoxyribonucleic acid |
| dpi | days post inoculation |
| EL | Eichorn and Lorenz |
| ET | Ethylene |
| EXT | Proline-rich extensin-like protein |
| F3H | Flavanone 3-hydroxylase |
| F5H | Ferulate 5-hydroxylate |
| FDR | False discovery rate |
| GA | Gibberellic acid |
| GAR2 | D-galacturonic acid reductase2 |
| GFP | Green fluorescent protein |
| GLP | Germin-like protein |
| GO | Gene ontology |
| GOX1 | Galactose oxidase |
| GPX3 | Glutathione peroxidase |
| GST1 | Glutathione S-transferase |
| Gt | Germ tube |
| hpi | Hour post inoculation |
| Hy | Hypha |
| IGS | Intergenic spacer region |
| JA | Jasmonic acid |
| LCC | Laccase |
| LGA1 | 2-keto-3-deoxy-L-galactonate aldolase |
| LGD1 | D-galactonate dehydrogenase |
| Ma | Multicellular appresoria |
| NaClO | Sodium hypochlorite |
| NW | Not washed |
| OAH | Oxaloacetate acetylhydrolase |
| PAL | Phenylalanine ammonia lyase |
| PCA | principal component analysis |
| PDA | Potato dextrose agar |
| PDB | Potato dextrose broth |
| PE | pectinesterases |
| PEL-like1 | Pectate lyase |
| PER | Peroxidase |
| pg | picogram |
| PG | Polygalacturonase |
| PLR | Pinoresinol/lariciresinol reductase |
| PR | Pathogenesis related |
| PRD1 | Dyp-type peroxidase |
| qPCR | Quantitative polymerase chain reaction |
| REViGO | Reduce + Visualize Gene Ontology |
| RLK | Receptor-like kinases |
| RNA | Ribonucleic acid |
| RNA-seq | RNA sequence |
| ROS | Reactive oxygen species |
| RS | Resveratrol synthase |
| SA | Salicylic acid |
| SIRD | Secoisolariciresinol dehydrogenase |
| SOD1 | Superoxide dismutase1 |
| SS | Surface sterlized |
| STS | Stilbene synthase |
| TF | Transcription factor |
| Trt | B. cinerea inoculated (Treated) |
| TUB | Tubulin |
| Vv | *Vitis vinifera* |
| W | Washed |
| WAK | Wall-associated kinase |
| wpi | weeks post inoculation |
| XYN11A | Endo-beta-1,4-xylanase |
| βGLUC | Beta-glucosidase 1 precursor |
| µg | Microgram |
| FAOSTAT | Food and Agriculture Organization of the United Nations Statistics Division |
| UHPLC-DAD-MS | Ultra High Performance Liquid Chromatography - Diode Array Detection - Mass Spectrometry |

## ABSTRACT

Grapes quality and yield are affected by bunch rot disease caused by the necrotrophic fungus *Botrytis cinerea*. Primary infections are mostly initiated at blooming by airborne conidia from overwintered sources. The fungus often remains quiescent from bloom until maturity and egresses at ripe where it causes bunch rot. Here, it is reported molecular analyses of the interaction between *B. cinerea* and the flower/berry of the cultivated grapevine (*Vitis vinifera* L.), in a controlled infection system, using confocal microscopy and integrated transcriptomic and metabolic analysis of the host and the pathogen. Open flowers from fruiting cuttings of the cv. Pinot Noir were infected with GFP labeled *B. cinerea* and samples taken at 24 and 96 hours post inoculation (hpi) (infected flowers), at 4 weeks post inoculation (wpi) (asymptomatic hard-green berries), and at 12 wpi (pre-egression and egression of the fungus on ripe berries) were studied. The observed penetration of the flower epidermis by *B. cinerea* coincided with increased expression of fungal genes encoding virulence factors, representing the effort of the pathogen to invade the host. Grapevine flowers responded with a rapid defense reaction involving genes associated with the accumulation of PR proteins, phenylpropanoids including 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 and a new status of asymptomatic coexistence is established. Afterwards, infected berries continued their developmental program without any visible symptom, although the presence of *B. cinerea* could be ascertained by plating out on selective media. Nonetheless, at the transcriptional level, the expressed quiescent fungal transcriptome highlighted that the fungus was modifying its cell wall to evade plant chitinases, besides maintaining basal metabolic activities. Also hard-green berries maintained activated response based on the expression of several PR family genes and genes involved in monolignol, flavonoid and stilbenoid biosynthesis pathways, in order to keep the pathogen quiescent. At 12 wpi, the transcripts of *B. cinerea* in the pre-egressed samples showed that virulence-related genes were expressed again, suggesting pathogenesis was resumed. The egressed *B. cinerea* expressed almost all virulence and growth related genes to enable the pathogen to colonize the berries. In response to egression, ripe berries reprogram different defense responses, though futilely. In conclusion, this study showed that the defense responses occurring in the grapevine flower and hard-green berry were able to restrict invasive fungal growth at the epidermal tissues, thus forcing the fungus to enter quiescence. However the pathogen was able to perceive and exploit ripening associated modifications, such as fruit's cell wall self-disassembly, and favorable external conditions, such as high humidity induced by cluster bagging practice, to recover an active metabolism and pathogenic activity, and eventually caused bunch rot.

# 1. GENERAL INTRODUCTION

## 1.1. Grapevine cultivation – production and uses

Grapevines (*Vitis* spp.) have evolved in several areas of the world, leading to the development of many different species including *V. labrusca* (L.) and *V. vinifera* (L.) (Mullins *et al*., 1992). The latter one being one of the most worldwide-grown perennial fruit crops whose origin of cultivation is probably in southern Caucasia, a region located on the border of Eastern Europe and Western Asia (Mullins *et al*., 1992). The other well-known species, *V. labrusca*, which is widely used for juice production, is native to North America (Creasy and Creasy, 2009). This species as other American species such as *V. riparia, V. berlandieri*, is much more resistant to pests and diseases than *V. vinifera* (Creasy and Creasy, 2009). Actually, many of the non *V. vinifera* species, which are usually not used for grape production, including *V. labrusca*, are used in breeding to confere resistance to soil-related conditions and pests for commercial cultivars (Creasy and Creasy, 2009). *V. vinifera* (here synonymously referred as grapevine) is a highly adaptable species. Cultivation limitations imposed by climatic conditions that are different from its center of origin are usually compensated by agronomic practices. Currently grapevine is cultivated throughout the world in a wide range of climates (from temperate to tropical climates), countries of the Mediterranean basin being the world's main producers (Bouquet, 2011). According to FAOSTAT (2014), the total world grapes production was estimated about 77 million tons in 2013, where Italy was the second top producer next to China (Food and Agriculture Organization of the United Nations Statistics Division, FAOSTAT, 2014, http://faostat3.fao.org/home/E).

The economic benefits obtained from grapevine derive from its berry. The nature of grape berry development is represented by two successive sigmoidal growth cycles, each with distinctive characterstics separated by a lag phase (Figure 1.1) (Coombe, 1992; Coombe and McCarthy, 2000). In the first cycle, berry formation, cell division and enlargement occur in pericarp tissue which determines a berry's final size and shape. The cycle lasts about 60 days after bloom, while the berry is hard and green and accumulates organic acids. The second cycle lasts from véraison (color change) to ripening. This cycle begins with the onset of berry softening, sugar accumulation, acids degradation, and berry colouring. Phenols and anthocyanin (in black-skinned

grapes) are also accumulated in this growth phase. Toward the end of the second growth cycle, aroma and flavor compounds are also produced.

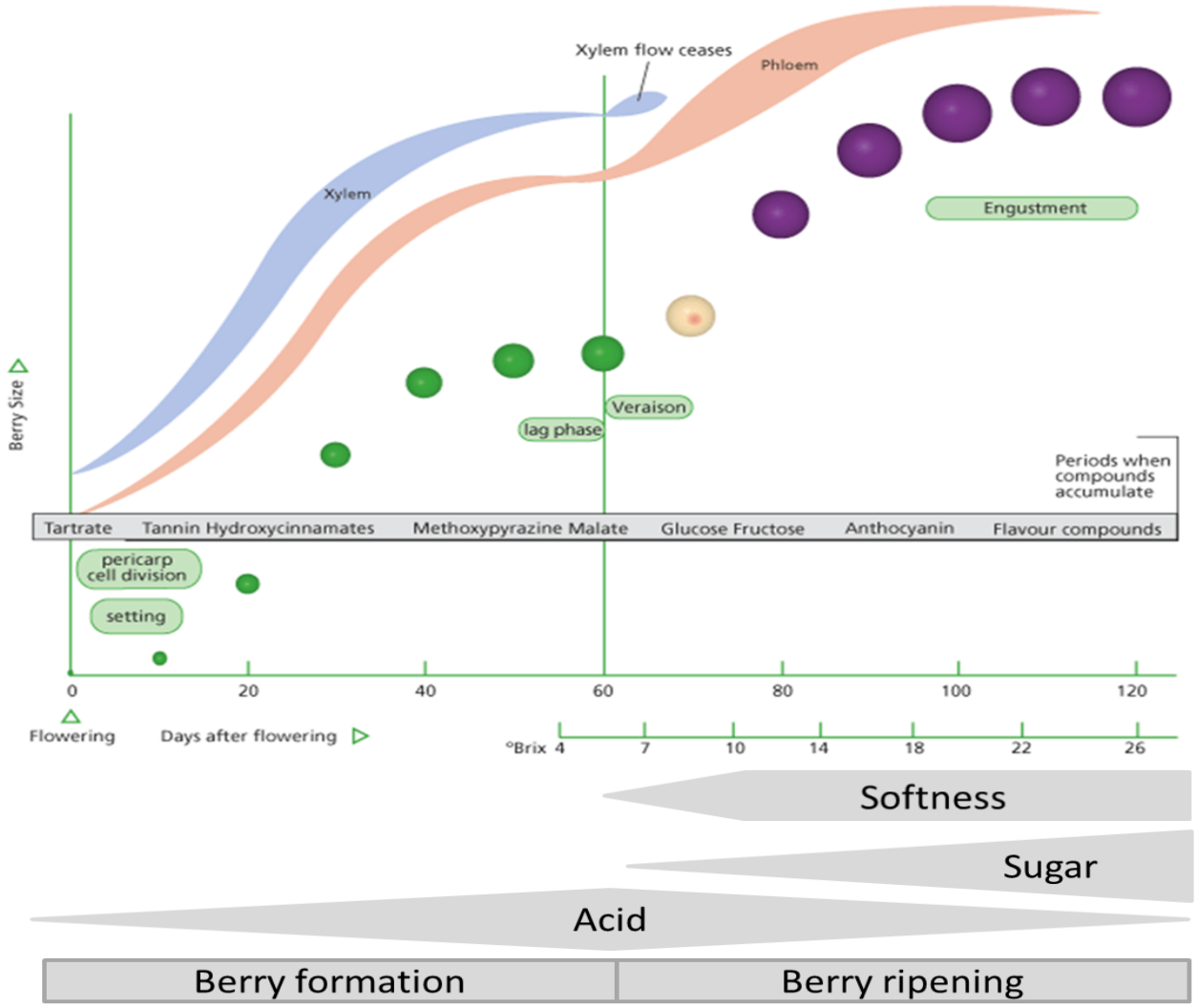


Figure 1.1. Diagram showing berry growth stages from flowering to ripening. Shown are: the relative size and color of berries at 10 days interval from flowering; periods when compounds accumulate; the level of juice brix; an indication of the rates of inflow of xylem and phloem vascular saps into the berry; and the rate of softness, sugar accumulation and acidity (Adopted from Kennedy, 2002)

The unique ability of grapevine berry to accumulate sugar, pectin (depending on cultivars), acids (particularly tartaric acid), and a wide range of aromatic compounds when ripe makes grapevine suitable for different uses (Creasy and Creasy, 2009). Major economic benefits come from wine and related fermented products, but also from fresh fruit, raisins, fruit juices and jams. To consumers, it has numerous nutritional and health benefits due to antioxidant polyphenols such as

flavonoids and resveratrol (Yadav et al., 2009). However, grapevine production can be jeopardized by adverse environmental conditions and biotic threats.

Disease and pest management consumes a large proportion of grapevine cultivation costs (Creasy and Creasy, 2009). Damage to grapevines can occur below and in the above-ground parts. Biotic stresses of grapes include diseases caused by bacteria, fungi, oomycets, viruses, and pests from insects, arthropods, birds, and mammals. The major diseases caused by fungi and oomycetes are briefly reviewed in the next section.

## 1.2. Grapevine diseases

Fungi and oomycetes represent the major cause of damage and losses in grape cultivation. The biotrophic oomycete pathogen, *Plasmopara viticola*, causes downy mildew in grapevine provoking severe leaf, shoot, and cluster damage. When leaves are infected, the pathogen causes a characteristic yellowish oily spots on the upper surface and massive sporulation on the underside, which looks like downy white (Pearson and Goheen, 1988; Ash, 2000). Infected berries and shoots have also a similar downy white appearance. Downy mildew is primarily a disease of warm and humid growing regions, and the absence of rainfall and high humidity reduces the spread of the disease (Pearson and Goheen, 1988). However, should there be a need to use fungicide, mostly copper-containing formulates are used (Ash, 2000).

Grapevine powdery mildew, also called oidium, is a devastating disease caused by *Erysiphe necator* (syn. *Uncinula necator*), an obligate biotroph fungus that can infect all grapevine green tissues (Gubler et al., 1999; Gadoury et al., 2012). Infection compromises photosynthesis and often leads to premature senescence and abscission of leaves; on berries it causes crack, acidity increase, and decrease anthocyanin and sugar content at ripe, with an overall impact on yield and quality (Lakso et al., 1982; Gubler et al., 1999; Calonnec et al., 2004). Most cultivated varieties are susceptible to powdery mildew, and growers make use of synthetic fungicides to control the disease (Gubler et al., 1999; Gadoury et al., 2012).

The ubiquitous *Botrytis cinerea* is another important grapevine's fungal pathogen, which causes *Botrytis* bunch rot or gray mold. This pathogen is the focus of this study, and is discussed in detail in the next section. Other grapevine diseases caused by fungal pathogens include cane and

leaf spot (*Phomopsis viticola*), dieback (*Eutypa lata*), black rot (G*uignardia bidwellii*), anthracnose (*Elsinoë ampelina*), and blackfoot disease (*Cylindrocarpon spp.*).

### 1.3. *Botrytis cinerea*

The genus *Botrytis* comprises about 28 species (Beever and Weeds 2004). However, with the exception of *B. cinerea*, most *Botrytis* species have a limited host range (Beever and Weeds, 2004). *Botrytis cinerea* (Pers.:Fries) represents one of the first described ascomycetes and is an economically relevant plant pathogen worldwide (Hennebert, 1973). Taxonomically, it is classified under kingdom: Fungi, phylum: Ascomycota, subphylum: Pezizomycotina, class: Leotiomycetes, order: Helotiales, family: Sclerotiniaceae, genus: *Botrytis*; and species: *Botrytis cinerea* (Williamson et al., 2007; Elad et al., 2016). Other species of *Botrytis* that are known to cause significant losses in crops are listed in Table 1.1.

Table 1.1. Lists of important *Botrytis* species with their hosts (Adopted from Dewey and Grant-Downton, 2016)

| Species | Date of description | Major plant host (genus/genera name) |
|---|---|---|
| *Botrytis aclada* | 1850 | *Allium* |
| *Botrytis allii* | 1917 | *Allium* |
| *Botrytis byssoidea* | 1925 | *Allium* |
| *Botrytis caroliniana* | 2012 | *Rubus, Fragaria* |
| *Botrytis cinerea* | 1794 | Multiple host |
| *Botrytis convoluta* | 1932 | *Iris* |
| *Botrytis deweyae* | 2014 | *Hemerocallis* |
| *Botrytis elliptica* | 1881 | *Lilium* |
| *Botrytis fabae* | 1929 | *Vicia* |
| *Botrytis fabiopsis* | 2010 | *Vicia* |
| *Botrytis gladiolorum* | 1941 | *Gladiolus* |
| *Botrytis globosa* | 1938 | *Allium* |
| *Botrytis hyacinthi* | 1928 | *Hyacinthus* |
| *Botrytis narcissicola* | 1906 | *Narcissus* |
| *Botrytis paeoniae* | 1897 | *Paeonia* |
| *Botrytis pelargonii* | 1949 | *Pelargonium* |
| *Botrytis polyblastis* | 1926 | *Narcissus* |
| *Botrytis porri* | 1949 | *Allium* |
| *Botrytis sinoallii* | 2010 | *Allium* |
| *Botrytis sinoviticola* | 2014 | *Vitis* |
| *Botrytis sphaerosperma* | 1949 | *Allium* |
| *Botrytis squamosa* | 1925 | *Allium* |
| *Botrytis tulipae* | 1913 | *Tulipa* |
| *Botrytis* sp. Group S | 2013 | *Fragaria, Vitis,* likely multiple others |

*B. cinerea* can undergo both sexual and asexual lifecycle. In the asexual cycle, the fungus produces numerous asexual conidia (macroconidia) that are borne at the tips of branching conidiophores, specialized hyphae (Figure 1.2). Conidia are short-lived propagules and their survival depends on temperature, moisture availability, sunlight exposure and microbial activity (Holz et al., 2004). They are metabolically dormant fungal structures and are considered as survival structures (Holz et al., 2004). *B. cinerea* can also produce temporary resting structures having thickened (hyaline) walls, called chlamydospores (Urbasch, 1983). Chlamydospores are often found in ageing cultures and suited to survive short drought periods (Urbasch 1983; Beever and Weeds, 2004). Another survival structures used for overwintering are sclerotia. These are structures resistant to adverse environmental condition, and are produced by melanized resting bodies (Holz et al., 2004).

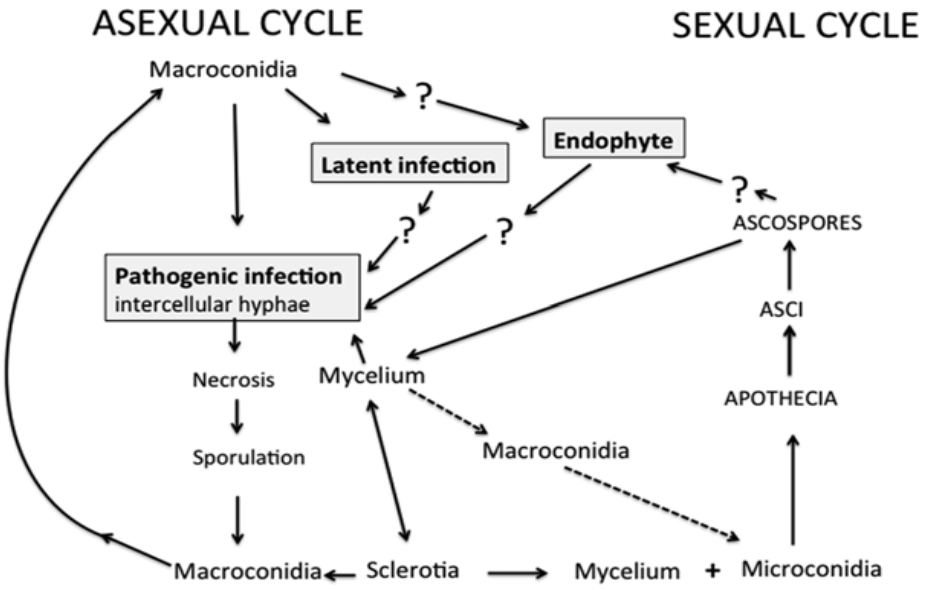


Figure 1.2. Diagram showing life cycle of *Botrytis cinerea* and interactions of different developmental stages in the life cycle (Adopted from Dewey and Grant-Downton, 2016). Macroconidia refer to conidia.

On the other hand, the fruiting structures in the sexual life cycle of *Botrytis* spp. are not commonly seen in nature (Lorbeer, 1980). Microconidia, are uninucleate and act as spermatia (Fukumori et al., 2004), are produced by macroconidia, old hyphae, and sclerotia (Lorenz and

Eichhorn, 1983; Fukumori et al., 2004) (Figure 1.2). The structure provides an alternative microscopic propagule when the fungus is under stress, in ageing culture or in cultures contaminated by other organisms (Holz et al., 2004). The fertilization of spermatia with receptive sclerotia give rise to apothecia from which asci, containing ascospores, are produced (Urbasch, 1983). Since most isolates of *B. cinerea* are naturally heterothallic, i.e. self-sterile, they can produce ascospore only when crossed with the opposite mating type, with the allele MAT-1 and MAT-2 being single mating type locus controlling sexual comapatibility (Faretra et al., 1988).

*B. cinerea* is an intriguing pathogen because of its unique characteristics: it can live as a pathogen (as necrotroph) but also as saprophyte or endophyte; it can be very devastating in some crops but it can also be of some benefit under certain conditions; it can cause early latent infection which damages the fruits mostly not before ripening (Rosslenbroich and Stuebler, 2000; Elad et al., 2004; Shaw et al., 2016). Upon conidial contact with plant tissues, germination and appresoria formation could possibly follow but without further invasion of the inner tissues (Williamson et al., 1987; Coertze and Holz, 2002). Such infections are described as quiescent or latent, where the fungus is arrested until favorable conditions are met, mostly when the tissue gets senescent (Shaw et al., 2016). There are also reports of *B. cinerea* colonizing host plants endophytically (Barnes and Shaw 2003; Sowley et al. 2010; Shaw et al., 2016). Endophytic *B. cinerea* can become infectious at a later stage of plant growth (maturity) or in storage or, can remain asymptomatic until the next cropping season, with a possibility to be transferred through clone or seed of the host (Barnes and Shaw 2003).

Undoutedly, *B. cinerea* is an important pre- and post-harvest pathogen, damaging plant products in field, storage, transport/transit, and on markets (Elad et al., 2004). The pathogen can attack more than 200 plant species (Jarvis, 1977). As many plant pathogenic fungi, short generation cycles and high amounts of progeny allow *B. cinerea* to colonize host plants rapidly and abundantly. The fungus causes blossom and leaf blights, bunch rot disease, and post-harvest fruit rots (Jarvis, 1977; Elad et al., 2004).

## 1.4. Grapevine and *Botrytis cinerea*

With regard to grapevine-*Botrytis* interaction, *B. cinerea* is part of most vineyards' natural microflora. The proposed life and disease cycles of the pathogen in vineyards is shown in Figure 1.3 (Elmer and Michailides, 2004). The fungus overwinters as sclerotia and/or mycelia in organic

debris in the vineyard. When the environmental conditions become suitable, the overwintered scerotia/mycelia produce conidia, which are usually a source of primary inoculum for pre-bloom and bloom infection (Elmer and Michailides, 2004). Grapevine cultivars greatly differ in their susceptibility to the *Botrytis* bunch rot disease (Creasy and Creasy, 2009). Cluster architecture, microclimate around the berry, and the content of preformed and inducible antifungal compounds (Langcake and McCarthy, 1979; Hill et al., 1981; Creasy and Coffee, 1988) are responsible for the varietal differences in *Botrytis* susceptibility.

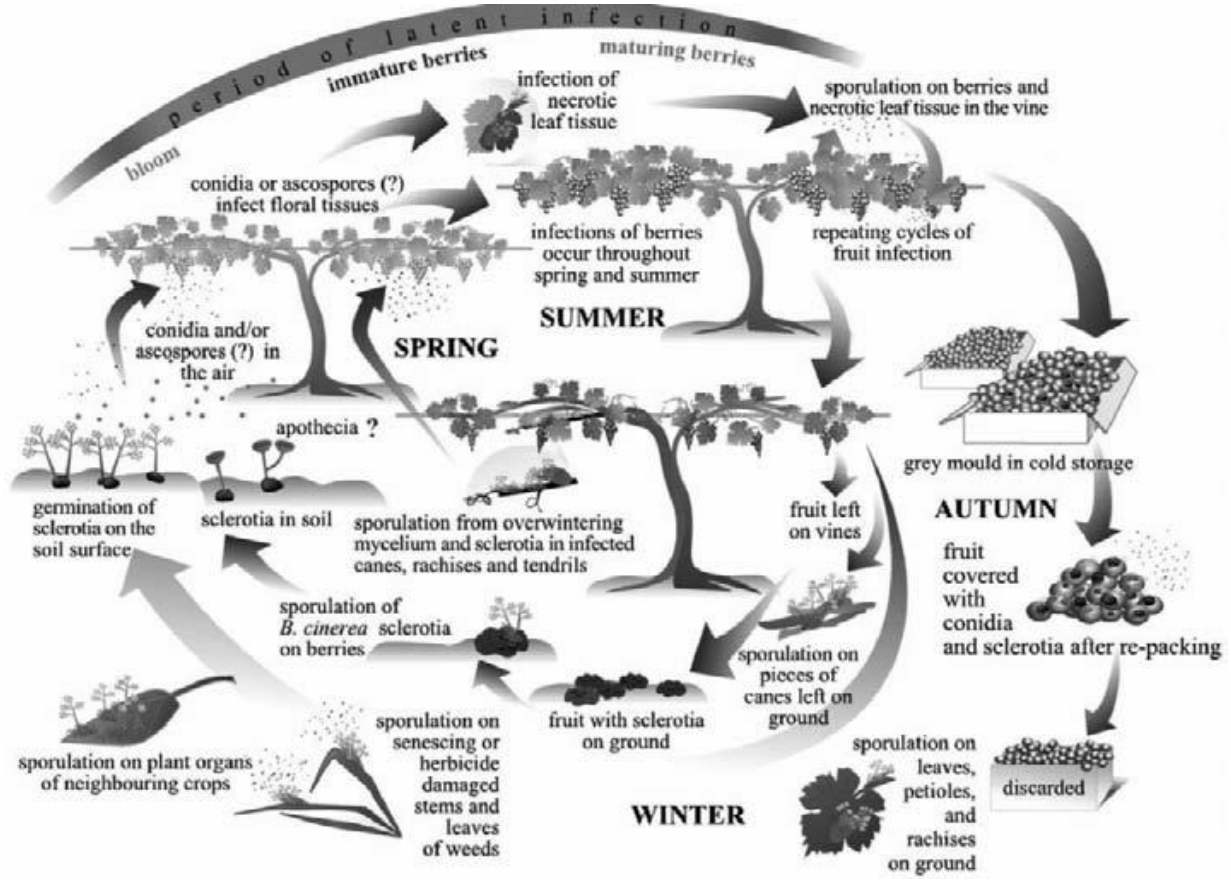


Figure 1.3. Life cycle of Botrytis cinerea and disease cycle of gray mold, as proposed by Elmer and Michailides (2004).

When infection happens at bloom, it generally remains quiescent until fruit ripening, and at ripening the pathogen resumes active growth to cause bunch rot. (McClellan and Hewitt, 1973; Nair et al., 1995; Keller et al., 2003; Pezet et al., 2003). Rainfall or long period of high humidity, cuticle cracking from pressure within the berry/cluster, and physical damages from insects, hail, and wind are environmental factors that dispose the outgrowth of the fungus causing ripe berries

bunch rot. Although *B. cinerea* is generally detrimental to grape quality by causing rotten berries in table grapes and off-flavors in wine (Loinger et al., 1977), for certain white-skinned cultivars, such as Sémillon, Riesling, Sauvignon Blanc, Muscadel, and Chenin Blanc under specific climatic conditions, it can be beneficial, as it happens in Tokaj (Hungary), Rheingau (Germany), and Sauternais (France) regions where enologists produce 'Botrytised' wine out of the rotten bunches, for this renamed 'noble rot' (Creasy and Creasy, 2009).

*B. cinerea* infection can possibly occur at any stage of fruit development, though primary infection often occurs at bloom time where symptoms are not apparent until berry ripening (McClellan and Hewitt, 1973; Nair et al., 1995; Keller et al., 2003). This delayed asymptomatic infection, whereby the infected tissue is not infectious, is known as quiescent or latent infection. Host factors that drive plant pathogens into quiescent stage are not fully known but limiting nutritional conditions, presence of preformed and inducible antifungal compounds, firm/thick cell wall, high production of reactive oxygen species, and inactivation of fungal pathogenicity factors are among the possible reasons (Prusky, 1996; Prusky et al., 2013). Besides these host factors, failure to produce adequate virulence factors by the pathogens drives them into quiescence. On the other hand, the physicochemical changes and gradual decline in antifungal compounds during ripening can lead to egression of quiescent pathogens (Prusky, 1996; Lattanzio et al., 2001).

In grapevine flowers inoculated with *B. cinerea* conidia, germ tubes were observed growing inside the narrow gap between calyx and ovary, that forms the receptacle area, and passing its quiescence there (Keller et al., 2003; Viret et al., 2004). According to Keller et al. (2003), higher disease severity at harvest was observed on bunches that received *B. cinerea* inoculation at flowering than controls, implying the importance of quiescent *B. cinerea* in determining the incidence of berry rot at ripe. In the epidemiology of *B. cinerea* in grape, flowering is an important stage as infection at this time is normally followed by quiescence. The asymptomatic presence of *B. cinerea* and its egression during ripening and/or post-harvest storage, when synthetic fungicides are not allowed, poses challenge in controlling the pathogen.

Knowledge of the molecular mechanisms involved during flower/berry-*Botrytis* interaction paves the way for devising appropriate control measure. Taking advantage of the availability of the genome sequences of *Vitis vinifera* (Jaillon et al., 2007; Velasco et al., 2007) and *B. cinerea* (Amselem et al., 2011; van Kan et al, 2016), a transcriptomic approach has been possible in this

study, allowing to unravel crosstalk between the pathogen and the plant at some key interaction stages of the infection process, i.e. at infection initiation, entry to quiescence, quiescence, and egression. All the infections have been conducted in controlled conditions in the greenhouse, using flowers raised from fruiting cuttings (as shown in Figure 1.4). In this way, it has been possible to overcome the seasonal requirements thus performing several experiments during the year, instead of one. Then, the use of the *Botrytis* strain expressing the fluorescent probe GFP would not have been allowed in the open field, due to law restrictions on the use of genetically modified organisms. Finally, *Botrytis* is naturally present in the field, so only using sterilized material grown in a protected environment would allow avoiding contamination during the experiment, which actually lasted from blooming to ripeness, i.e. more than 10 weeks.

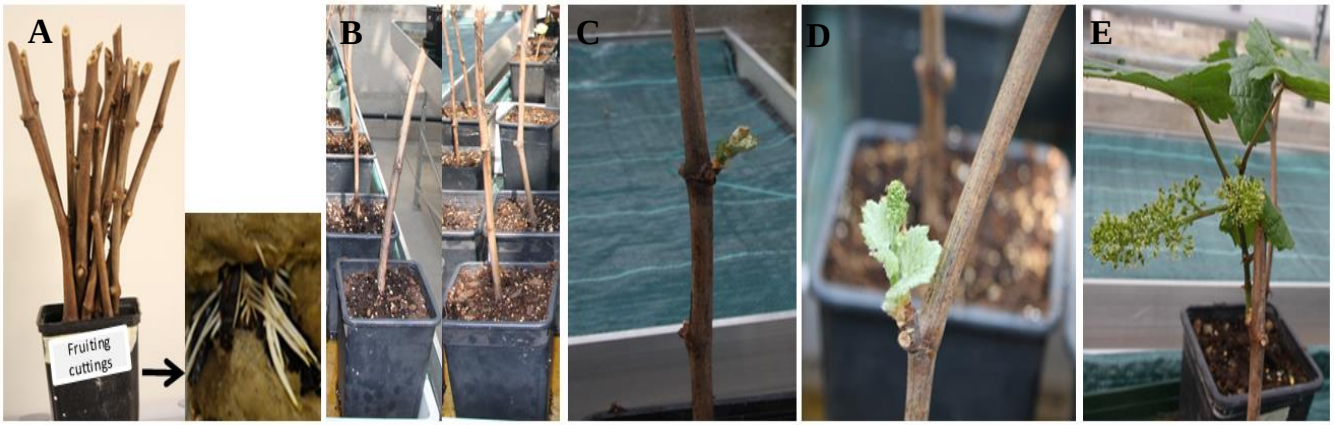


Figure 1.4. Grapevine flowers raised from fruiting cuttings. A, Fruiting cuttings in rooting pot with moistened rock wool in cold room (4 °C) on heat mat, to stimulate rooting while keeping the above buds dormient; adventitious roots after 6 weeks. B, Fruiting cuttings, with adventitious roots, transferred into pots with growing mix to greenhouse. C, Budburst, 1-2 week after being transferred to greenhouse. D, Emerging inflorescence after apical meristem was removed, 2-3 weeks after being transferred to greenhouse. E, Inflorescence with single flowers separated, 3-4 weeks after being transferred to greenhouse.

## 1.4. OBJECTIVE

The aim of this research was to understand the interaction between *B. cinerea* and grapevine inflorescences at molecular level using integrated approaches (confocal microscopy, transcriptome and metabolome approaches). The first objective was to understand the crosstalk between grapevine inflorescence and *B. cinerea* at the initiation of infection and entry to quiescence state. The second one was to understand the interaction between quiescent *B. cinerea* and hard-green berry and between egressed *B. cinerea* and ripe berry.

The results obtained will be described in two main chapters, responding to the two above mentioned objectives: i) Grapevine flower and *Botrytis cinerea* interaction and ii) Interaction between grape berry and *B. cinerea* during quiescent and egression infection stages. These two chapters correspond to two paper works, the first is already submitted to Plant Cell & Environment Journal and the second is under preparation

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# 2. GRAPEVINE FLOWER AND *Botrytis cinerea* INTERACTION

## 2.1. Abstract

Grapes quality and yield can be severely impaired by bunch rot, caused by the necrotrophic fungus *Botrytis cinerea*. Infection often occurs at flowering and the pathogen stays quiescent until fruit maturity, when symptoms become evident. Here, we report a molecular analysis of the early interaction between *B. cinerea* and the flower of the cultivated grapevine (*Vitis vinifera* L.), using a controlled infection system, confocal microscopy and integrated transcriptomic and metabolic analysis of the host and the pathogen. Flowers from fruiting cuttings of the cv. Pinot Noir were infected with GFP labeled *B. cinerea* and studied at 24 and 96 hours post inoculation (hpi). We observed that penetration of the epidermis by *B. cinerea* coincided with increased expression of genes encoding cell wall degrading enzymes, phytotoxic secondary metabolites, and proteases. Grapevine responds with a rapid defense reaction involving 1193 genes associated with the accumulation of antimicrobial proteins, phenylpropanoids including stilbenoids, reactive oxygen species and cell wall reinforcement. At 96 hpi the reaction appears largely diminished both in the host and in the pathogen. Afterwards, berries continue their developmental program up to véraison without any visible symptom, whereas the presence of *B. cinerea* can be ascertained by plating out on selective media. Our data indicate that the defense responses occurring in the grapevine flower within 24 hours after *B. cinerea* inoculation collectively are able to restrict invasive fungal growth into the underlying tissues, thereby forcing the fungus to enter quiescence until the conditions become more favorable to resume pathogenic development.

## 2.2. Introduction

Grapevine yield and quality faces challenges worldwide from biotic stresses, mainly caused by fungi and oomycetes like *Botrytis cinerea, Plasmopara viticola*, and *Erysiphe necator*. *B. cinerea*, a necrotroph responsible for pre- and post-harvest disease in a wide range of crops, causes bunch rot in grapevine. In vineyards, *B. cinerea* is part of the natural microflora where primary infections of berries are usually initiated by airborne conidia from overwintering sources (Nair et al., 1995; Elmer and Michailides, 2004). Bunch rot frequently occurs on ripe berries close to harvest. Wet conditions together with damage to ripe berries, due to cuticle cracking from pressure within the berry/cluster and physical damage from biotic and abiotic sources occurring during ripening, the expression of bunch rot even though the primary infection could have occurred at earlier stages of berry development (McClellan and Hewitt, 1973; Nair et al., 1995). Bunches inoculated at flowering with *B. cinerea* were reported to have higher disease severity at maturity (Keller et al., 2003; Pezet et al., 2003b), implying that bunch rot disease observed during ripening may not only be due to *de novo* infection, but also due to latent infections that occurred at earlier stages of berry development. A similar infection strategy of the pathogen was also observed in strawberries and raspberries (Jarvis, 1962; Williamson et al., 1987; Jersch et al., 1989). This delayed asymptomatic infection is known as quiescent infection.

Usually the cosmopolitan *B. cinerea*, upon contact with the host, incites cell death by producing phytotoxins and cell wall degrading enzymes and manipulates hosts metabolisms to facilitate colonization (van Kan, 2006; Choquer et al., 2007; Williamson et al., 2007). A deviation from this common necrotrophic lifestyle, where *B. cinerea* behaves as a facultative endophyte has also been observed (Williamson et al., 1987; McNicol and Williamson, 1989; Coertze and Holz, 2002; Shaw et al., 2016). This symptomless colonization, caused by a long-lived physiological switch (van Kan et al., 2014), which can shift to active and symptomatic behavior under favorable conditions, differentiates the endophytic lifestyle from the relentless necrotrophic infection behavior.

In grapevine, *B. cinerea* infection often occurs at blooming and then remains quiescent until ripening (McClellan and Hewitt, 1973; Nair et al., 1995; Keller et al., 2003; Pezet et al., 2003b). Berry developmental stages between bloom and véraison are mostly resistant to *B. cinerea* infection. Such development related resistance could be linked to preformed and inducible

antifungal compounds, as well as skin features of immature berries. Phenylpropanoid and flavonoid extracts of young berries, as well as resveratrol, can inhibit *B. cinerea* growth i*n vitro* (Goetz et al., 1999; Schouten et al., 2002b; Pezet et al., 2003b). Furthermore, polyphenols in the berry skin cell wall and the thickness of epidermal cell layer complex were reported among the resistance factors (Mlikota-Gabler et al. 2003; Deytieux-Belleau et al., 2009). More recently, Agudelo-Romero et al. (2015) reported a large transcriptional activation of genes related to secondary metabolism and hormonal signaling (jasmonic acid [JA], ethylene [ET], and auxins) upon *B. cinerea* infection of immature berries of cv. Trincadeira. Another study on grapes infected at véraison reported the accumulation of reactive oxygen species (ROS), the activation of the salicylic acid (SA) dependent pathway and the induction of stilbene and lignin biosynthesis as defense mechanisms to arrest *B. cinerea* progression (Kelloniemi et al., 2015).

In disease management, quiescent infection has important implications for proper timing of prophylactic measures, to reduce stresses factors that may trigger egression of the quiescent pathogen, and to prolong quiescence to the point where the produce is not affected even after harvest (Jarvis, 1994). Concerning grapevine, flowering is an important stage in the epidemiology of *B. cinerea* as infection at this stage is followed by quiescence. Therefore, understanding the interaction between *B. cinerea* and grapevine inflorescence is vital to implement proper management in order to limit consequent yield losses. Despite this, knowledge about the molecular mechanisms of the interplay between *B. cinerea* and grapevine inflorescences at bloom is lacking. Taking advantage of the availability of the genome sequences of *Vitis vinifera* (Jaillon et al., 2007; Velasco et al., 2007) and *B. cinerea* (Amselem et al., 2011; van Kan et al, 2016), we analyzed the transcriptional alterations of both organisms during flower infection to understand the molecular mechanisms associated with the early stage of this interaction. Microscopic observation and metabolic profiles were combined with the transcriptomic analyses to further our understanding of the infection process at infection initiation and initial fungal quiescent stages.

## 2.3. Materials and methods

### 2.3.1. Plant material and *Botrytis cinerea* inoculation

Winter woody cuttings were collected from an experimental vineyard (*Vitis vinifera* cv. Pinot Noir) of the Fondazione Edmund Mach, Trentino-Alto Adige, Italy and stored at 4 °C until use. Flowers were raised from the cuttings following the technique of Mullins and Rajaskekaren

(1981). At the stage when single flowers of an inflorescence separate (EL17, according to Eichorn and Lorenz [1977]), they were thinned in order to have a manageable number of flowers per inflorescence to ensure that each flower could receive *B. cinerea* conidia. Cuttings were grown in a growth chamber at 24°C, with a 16 h light cycle.

Transgenic grapevine plants (*Vitis vinifera,* Microvine mutant) harboring the $H_2O_2$-specific HyPer probe, targeted to the cytosol, were generated using the DNA construct described in Costa el al. (2010).

*Botrytis cinerea* (isolate B05.10) was cultured on potato dextrose agar (PDA) in Petri dishes and incubated at 25 °C. After 10 days, conidia were harvested in distilled water and conidia concentration was determined under light microscope using a hemacytometer. At full cap-fall stage (EL25/26), each flower was inoculated by positioning 1.5 µl of a $2 * 10^5$ $ml^{-1}$ conidia solution close to receptacle area. After inoculation, the whole cutting was immediately bagged in water sprayed, clear plastic bag for 24 h in order to ensure high humidity, an essential factor for conidial germination. For microscopic observation and post-inoculation evaluation (plating out test), a genetically transformed strain of B05.10 expressing a green fluorescent protein (GFP) was used due to its fluorescent signal and ability to grow on selective medium (PDA with70 µg/ml Hygromycin B).

### 2.3.2. Microscopic observations and detection of quiescent *Botrytis cinerea*

Confocal laser scanning microscopy analyses were performed using a Leica SP5 imaging system (Leica Microsystems, D-68165 Mannheim, Germany) and a Zeiss LSM700 (Carl Zeiss Microscopy, Germany). GFP and chlorophyll were excited at 488 nm and the emission was collected at 515-560 nm and 650-750 nm, respectively. For HyPer detection confocal microscopy analyses were performed according to Costa et al. (2010). Thin slices of fertilized gynoecia, which were manually cut from inoculated flowers, were subjected to microscopic observation.

For quiescent *B. cinerea* detection, the plating out method on selective medium was used. Eight fruitlets from each of 6 biological replicates were sampled daily from 1 to 7 and at 14 days post inoculation. Fruitlets were incubated on PDA with hygromycin at room temperature for a week before or after washing, or after surface sterilization. Washing was with sterile water, three rinses of 1 minute each with gentle shaking; whereas surface sterilization was carried out with 70% ethanol (1 min) followed by 1% (vol/vol) NaClO (3 min) and three rinses in sterile water (Keller

et al., 2003). Appearance of mycelial growth from healthy-looking fruitlets was scored as confirmation of quiescent *B. cinerea* on the fruitlets. Fruitlets > 4 mm in diameter (approximately) were cut into half before plating. Statistical significance among the treatments was calculated by Tukey's Honestly Significant Difference test on square root transformed data.

### 2.3.3. Secondary metabolites and RNA extraction

Inflorescences from fruiting cuttings that were either mock (control) or B05.10-conidia inoculated at cap-off stage, were collected at 12, 24, 48, 72 and 96 hpi, in three biological replicates, immediately frozen in liquid nitrogen, and kept at -80 °C until use. A biological replicate, throughout this study, is an inflorescence from a fruiting cutting. The samples used for polyphenol and RNA extraction were independent. Prior to polyphenol and RNA extraction, the samples were ground in liquid nitrogen. RNA was extracted using Plant Total RNA Kit (Sigma-Aldrich) following the manufacturer's protocol. For targeted secondary metabolite analysis, sample preparation and Ultra High Performance Liquid Chromatography - Diode Array Detection - Mass Spectrometry (UHPLC-DAD-MS) analysis were conducted as described in Vrhovsek et al. (2012).

For *B. cinerea* RNA extraction, B05.10 conidia were incubated in flask with potato dextrose broth (PDB) for 12 hours (Supplemental Figure S2.1) with 30 rpm shake in three biological replicates. Conidia obtained from a Petri dish were considered as a biological replicate.

### 2.3.4. RNA sequencing, data processing and data analysis

Three biological replicates harvested at 24 and 96 hpi were used for RNA-Seq analysis. Approximately 20 million strand-specific, 100 bp long sequences were obtained for each sample using a Next Generation Sequencing Platform HiSeq 1500 (Illumina, San Diego, CA). The quality of the the Illumina single-end reads was checked using FastQC (version 0.11.2) software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and pre-processed for adapter with cutadapt (version 1.8.1) (Martin, 2011). The resulting reads were aligned separately to the *B.cinerea* (strain B05.10) (http://fungi.ensembl.org) and grapevine (12Xv1, http://genomes.cribi.unipd.it/) genomes using the Subread aligner (Liao et al., 2013). Raw read counts were extracted from the Subread alignments using the featureCount read summarization program (Liao et al., 2014).

For grapevine, differential expression analysis of genes was performed taking advantage of the voom method (Law et al., 2014) which estimates the mean-variance relationship of the log-counts, generating a precision weight for each observation that is fed into the limma empirical Bayes analysis pipeline (Smyth, 2004). Genes were considered differentially expressed if they fulfill a *p*-value <0.05 and an absolute fold change of ≥1.5. 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). Enriched GO terms (FDR <0.05) were visualized using the 'Reduce + Visualize Gene Ontology' (REViGO) webserver (http://revigo.irb.hr; Supek et al., 2011). Furthermore, the grapevine molecular network gene annotation, VitisNet, (Grimplet et al., 2012) was also used to identify enriched molecular networks ($P < 0.05$) using VESPUCCI (http://vespucci.colombos.fmach.it) (Moretto et al., 2016). MapMan tool (Thimm et al., 2004) was used to visualize differentially expressed genes in the context of biotic stress pathway using the GrapeGen 12Xv1 annotations version (Lijavetzky et al., 2012) as MapMan Bins. For *B. cinerea*, gene set enrichment analysis was performed using Fisher's exact test.

### 2.3.5. Quantitative polymerase chain reaction (qPCR)

For qPCR assays, cDNA was synthesized from 3 µg of the same RNA used for RNA-Seq analysis, treated with DNase I (Ambion), using the SuperScript™VILO™cDNA Synthesis Kit (Invitrogen). qPCR was performed in a Viia7 thermocycler (Applied Biosytems) 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 (Kapa Biosystems) using the standard fast protocol. Each amplification reaction was run in triplicate. For normalization, *VvACT* and *VvTUB*, and *BcRPL5* and *BcTUBA* genes were selected using GeNORM (Vandesompele et al., 2002) as reference for grapevine and *B. cinerea*, respectively. Amplification efficiencies of each primer pair were calculated with LinReg software (Ruijter et al., 2009). The obtained amplification efficiency was used to calculate the relative quantity (RQ) and normalized relative quantity (NRQ) according to Hellemans et al. (2007). Statistical analyses of the qPCR results were made after $\log_2$(NRQ) transformation (Rieu and Powers, 2009). All primers and corresponding gene identifiers can be found in Supplemental Table S2.1. Statistical significance was calculated by Tukey's Honestly Significant Difference test or an unpaired heteroscedastic Student's *t* test, considering each technical replicate as an individual sample.

### 2.3.6. DNA extraction, standard curve and DNA quantification

DNA was isolated from grapevine flowers and *B. cinerea* (strain B05.10) mycelium using the Dneasy Plant Minikit (Qiagen) following the manufacturer's protocol. DNA from mycelium, obtained from conidia incubated for 48 h as mentioned above, and uninoculated grapevine flower were used to generate calibration curves to estimate the amount of fungal DNA in inoculated samples, 12, 24, 48, and 96 hpi, and 1 wpi. The inoculated samples were replicated three times. From 1 wpi samples, RNA was also extracted following the technique mentioned above. The samples used were independent from those used for RNA-Seq and secondary metabolite studies.

Genomic DNA was used as a template for qPCR with similar amplification procedure described above using primers Bc3, ribosomal IGS spacer, and VvRS I, resveratrol synthase gene I. For the standard curve, qPCR reaction were carried out in triplicate from known fungal or plant DNA extracts which were serially diluted 5 times. The standard curves were generated by plotting the log of DNA (pg) against the Ct value (Supplemental Figure S2.2). The Ct values obtained from inoculated samples were used to extrapolate the amount of genomic DNA from the standard curves. Genomic DNA of *B. cinerea* in a sample was normalized to the amount of grapevine genomic DNA in that sample.

## 2.4. Results

### 2.4.1. *Botrytis cinerea* infection of grapevine flower

Artificial infection of grapevine flowers with GFP-labeled B05.10 strain was conducted at full cap-off stage (EL25/26) (Figure 2.1A). The infection was monitored for two weeks post inoculation (wpi) and during this period there were no visible symptoms of infection or fungal growth (Figure 2.1B-E). Although fungal conidia germination, formation of appressoria and penetration into the flower cuticle on the gynoecium above the floral disc were observed 24 hours post inoculation (hpi) by confocal microscopy following the GFP signal of *B. cinerea* (Figure 2.1 F and Supplemental Figure S2.3), no substantial progress in fungal growth was appreciated at 96 hpi (Figure 2.1G). The presence of viable fungus during 2 wpi was confirmed by plating out experiments. Inoculated but healthy-looking fruitlets were incubated on selective media to allow the growth of the GFP-labeled *Botrytis* strain only. Before plating, fruitlets were either washed with distilled water or surface sterilized, to discriminate respectively either non germinated conidia or germinated conidia laying on the external layer of the flower tissue, from those bearing

penetration structures and grown in the inner layers. Germinated *B. cinerea* conidia were present on the skin of 90 % of the inoculated fruitlets, whereas about 30 % of the samples showed the presence of *B. cinerea* below the external cell layers of the fruitlet (Figure 2.1H). A preliminary test confirmed that washing within 6 hpi was able to remove ungerminated conidia from flowers and that surface sterilization abolished *B. cinerea* viability (Supplemental Figure S2.4).

To check if a similar load of *B. cinerea* was present at different post inoculation times, the amount of fungal DNA was estimated by quantifying the ribosomal IGS DNA (Supplemental Figure S2.2A), while the amount of grapevine DNA was also estimated by amplification of the resveratrol synthase gene I (Supplemental Figure S2.2B). As shown in Figure 2.2A, the relative amount of fungal DNA compared to plant DNA ranged from 4 to 6%, with a slight increase within two dpi, indicating initial pathogen growth, followed by a slight decrease possibly associated to a quiescent state. Furthermore, the expression profile of *B. cinerea* actin, *BcACTA*, an indicator of active growth, confirmed that the growth of the fungus *in planta* was relatively high up to two dpi, and then decreased slightly but significantly, supporting pathogen quiescence (Figure 2.2B).

Inoculated inflorescences were inspected until fruit ripening. At full coloring (approximately 10 wpi), bunches were either bagged with plastic bags, to create favorable humidity for *B. cinerea*, or left as such. About two weeks after bagging, egression of *B. cinerea* was observed on about 40 % (39 ± 9 %) of the inoculated berries (Supplemental Figure S2.5A). Cross checking of the strain using fluorescence microscope on mass of mycelia taken from the rotting bunch confirmed that the strain was the GFP-labeled B05.10 inoculated at cap-off stage (Supplemental Figure S2.5B). On the other hand, no egression was observed from unbagged bunches. In addition, to see if bagging can also trigger egression before maturity, bunches at pepper-corn satge, which were infected at cap-off stage, were bagged for two weeks, but no *B. cinerea* egression was observed (Supplemental Figure S2.6).

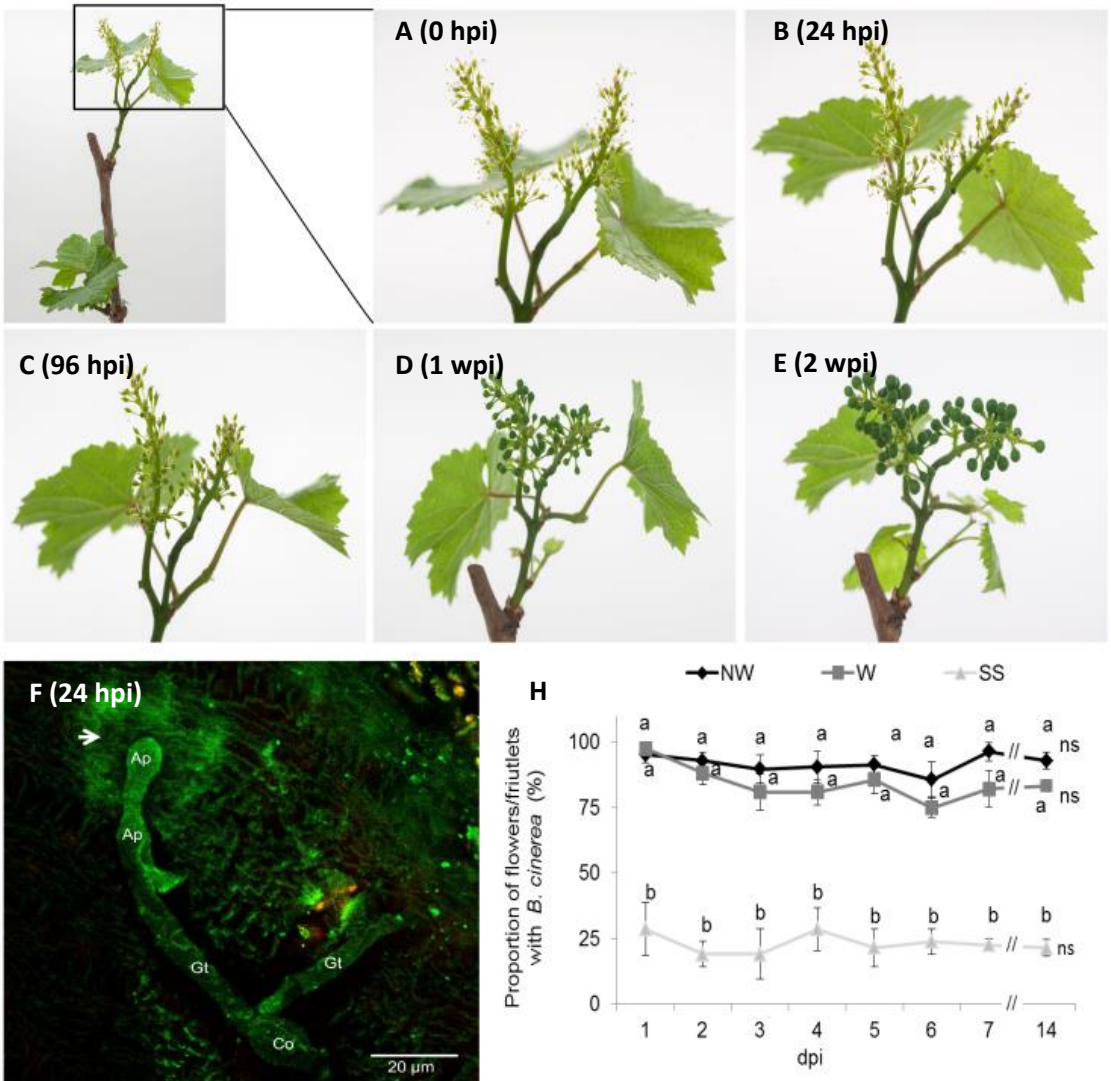



Figure 2.1. Grapevine flowers infected with *Botrytis cinerea*. Flowers inoculated with GFP-labeled B05.10 strain at full cap-fall stage (EL25/26), 0 hpi (A), and asymptomatic inflorescence up to two weeks post inoculation – B to E. F and G, confocal microscope images of GFP-labeled B05.10 infecting the cuticle of a grapevine flower 24 and 96 hours after inoculation. Ap, appressoria; Co, conidium; Gt, germ tube; Hy, hypha. Autofluorescence around penetration site (shown by arrow) indicates cell wall fortification. H, Plating out of infected fruitlets on selective media (PDA with Hygromycin B, 70 µg/ml) to check the presence of quiescent *B. cinerea* on infected fruitlets before (NW) or after washing (W), or after surface sterilization (SS). dpi, dpi, days post inoculation. Values at each day represent mean proportion of fruitlets (eight fruitlets from each of 6 biological replicate considered) showing GFP-labeled B05.10 growth on the selective media. Error bars indicate standard error. Mean proportions followed by a common letter within a dpi are significantly not different among NW, W and SS, according to Tukey's Honestly Significant Difference test ($P \leq 0.05$), using one way ANOVA. The mean proportions throughout the two weeks

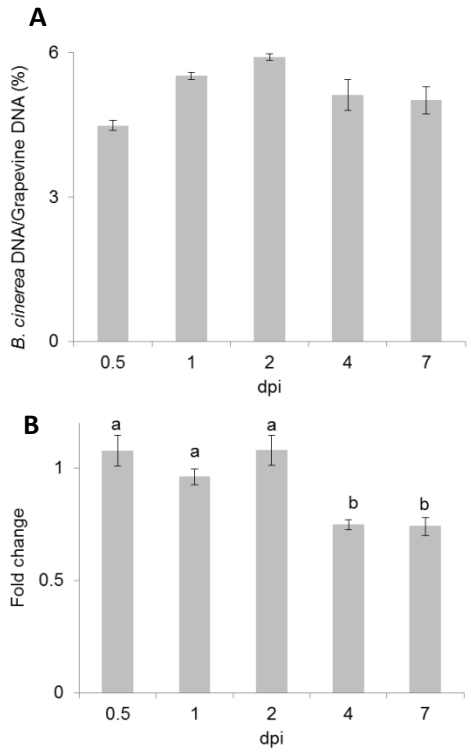



Figure 2.3. Relative quantification of genomic DNA (A) and expression profile of actin gene (B) from *B. cinerea* at different days post inoculation (dpi). A, Amount of *B. cinerea* gDNA relative to grapevine gDNA, measured by amplification of the *B. cinerea* gene *Bc3* (ribosomal IGS spacer) and the grapevine gene *VvRS I* (resveratrol synthase gene I) on gDNA from *Botrytis*-inoculated flowers. The Kruskal-Wallis one way ANOVA test revealed that the amount of *B. cinerea* gDNA at different days post inoculation (dpi) is not significantly different, $P = 0.123$. Error bar represents standard error of mean of three biological replicates. B, Relative expression of a *B. cinerea* actin gene, *BcACTA*, to monitor the growth of the pathogen *in planta*. Bars represent fold change of inoculated samples relative to PDB cultured *B. cinerea* (control). Normalization based on the expression levels of ribosomal protein L5, *BcRPL5*, and α tubulin, *BcTUB*A, was carried out before calculating fold changes. Expression values followed by the same letter are significantly not different between samples, according to Tukey's Honestly Significant Difference test ($P \leq 0.05$), using one way ANOVA on log2(NRQ).

### 2.4.2. Transcriptome analysis of infected grapevine flowers

Three biological replicates of mock or B05.10 inoculated flowers were harvested at 24 and 96 hpi for dual (plant and fungus) transcriptome analysis using RNA-seq. These time points were chosen to understand the process of infection initiation (24 hpi) and progress (96 hpi), if any. As a control sample for *Botrytis*, an *in vitro* grown culture was used. The high quality trimmed reads obtained from the RNA-seq experiment were aligned against the 12Xv1 gene prediction of *V. vinifera* genome (http://genomes.cribi.unipd.it/grape/) and the *B. cinerea* strain B05.10 genome (http://fungi.ensembl.org). The fraction of reads from *Botrytis*- and mock-inoculated flowers mapped to the *V. vinifera* reference genome was between 65 and 82 %, whereas only up to 4.6 % could be mapped to the *B. cinerea* genome (Supplemental Table S2.2). In the case of *B. cinerea*, cultured in PDB, a much larger proportion of reads was mapped to the fungal genome (about 90%), suggesting that the scarce number of fungus reads derived from the infected flowers is likely caused by low number of conidia used for the infection (around 300) and of the limited fungal growth after inoculation.

As for grapevine, the biological variability within replicates and among experimental conditions was analyzed by principal component analysis (PCA). As shown in Figure 2.3A, the first principal component, which explains 38.4% of the variance, separates the two time-points (24

and 96 hpi) and also the 24-hours' *Botrytis* treated vs. untreated samples. In contrast, all samples collected at 96 hpi seem very similar at a whole transcriptome level, as indicated by the large overlap in the PCA.

Differential expression of grapevine genes was calculated between *Botrytis*- vs. mock-inoculated flowers within each time point after *t*-test, imposing an absolute minimum fold-change of 1.5 and a *p*-value < 0.05. At 24 hpi, 1193 genes were differentially expressed (upregulated or downregulated), whereas at 96 hpi only 265 genes were differentially expressed (Figure 2.3B, Supplemental Figure S2.7 and Supplemental Table S2.3). The overlap between the two sets was limited to 49 upregulated and 4 downregulated genes (Figure 2.3C). 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.

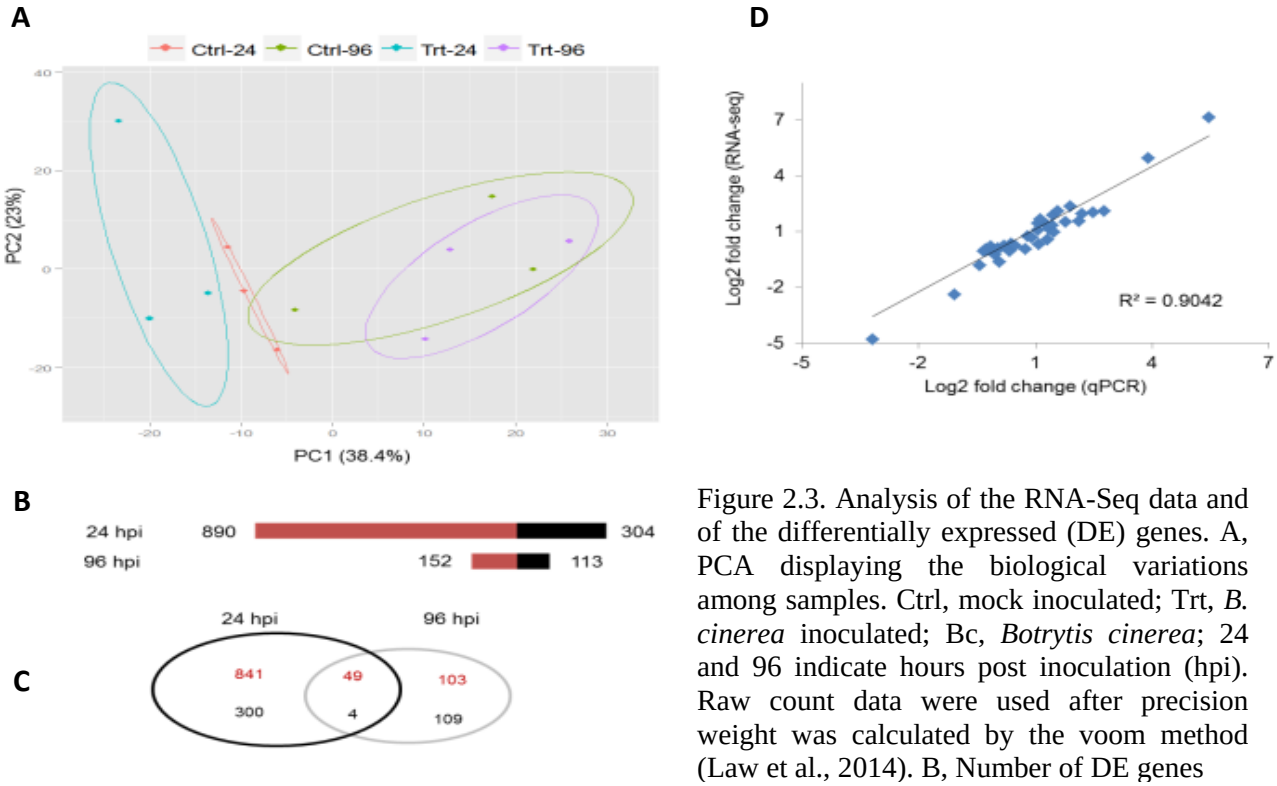



Figure 2.3. Analysis of the RNA-Seq data and of the differentially expressed (DE) genes. A, PCA displaying the biological variations among samples. Ctrl, mock inoculated; Trt, *B. cinerea* inoculated; Bc, *Botrytis cinerea*; 24 and 96 indicate hours post inoculation (hpi). Raw count data were used after precision weight was calculated by the voom method (Law et al., 2014). B, Number of DE genes ($P$ < 0.05, absolute fold change > 1.5) upon *B. cinerea* infection at 24 and 96 hpi; upregulated genes (red) and downregulated genes (black). C, Venn diagram showing the number of DE genes unique or common to 24 and 96 hpi. D, Validation of RNA-Seq data by qPCR assay: correlation of fold change values for 20 *Vitis* genes obtained by RNA-Seq and qPCR.

Gene expression values from RNA-Seq analysis were validated using qPCR assay. The expression measurement of 20 grapevine genes (Supplemental Table S2.2) by qPCR was in very good agreement ($R^2$ >0.90) with the results obtained by RNA-Seq (Figure 2.3D).

### 2.4.3. Defense related responses are largely induced in the flower upon *Botrytis cinerea* infection

Differentially expressed (DE) genes were annotated according to two different publicly available databases for grapevine, namely Gene Ontology (GO) (http://genomes.cribi.unipd.it/grape/) and VitisNet (https://www.sdstate.edu/ps/research/vitis/pathways.cfm), in order to characterize them in a comprehensive manner. Functional class enrichment analyses performed on the GO and VitisNet databases provided consistent and complementary results (Table 2.1 and Supplemental Table S2.5). A clear regulation of those classes typically modulated during biotic stress responses was found, they are represented in the MapMan pathway depicted in Figure 2.4 and significantly enriched in *Botrytis*-infected flowers (Supplemental Table S2.6). In the following text, these classes will be presented in detail.

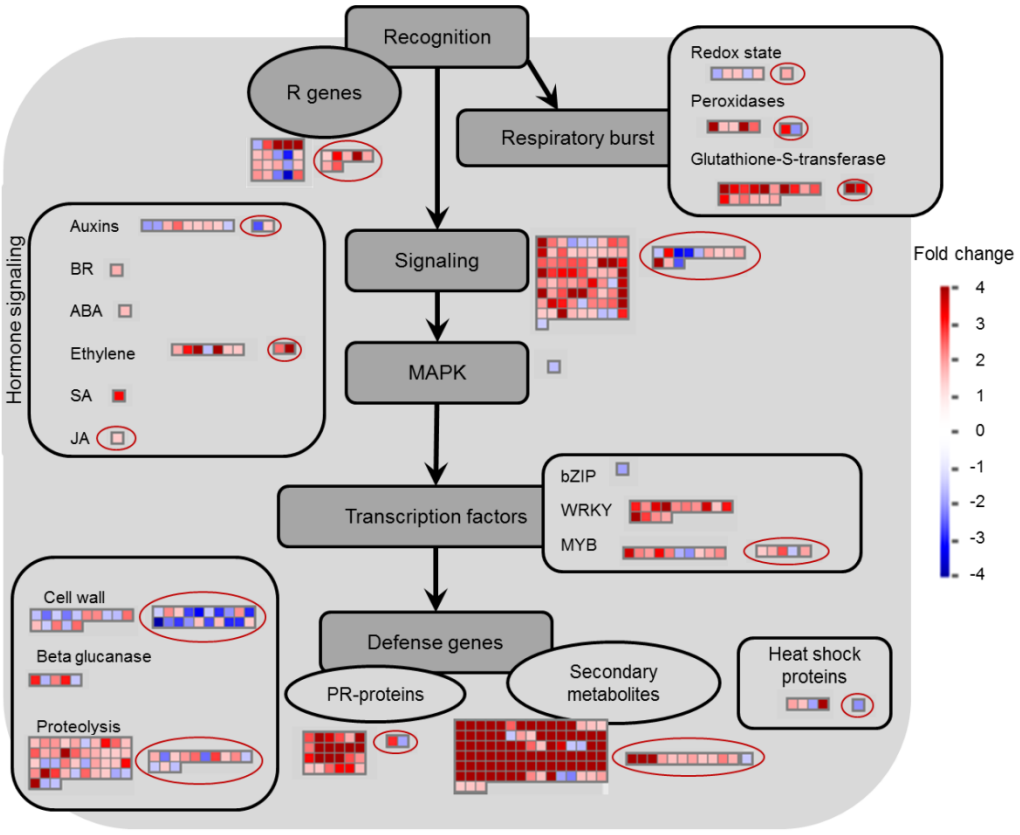



Figure 2.4. MapMan overview of biotic stress in inoculated flower at 24 and 96 hpi (red circled). Upregulated and downregulated genes are shown in red and blue, respectively. The scale bar displays fold change values. ABA, abscisic acid; BR, brassinosteroid; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; SA, salicylic acid. The list of MapMan enriched pathways within DE genes is provided in Supplemental Table S2.6.

Table 2.1. Enriched Grapevine Molecular Networks according to VitisNet annotation

| VVID | Network Name | Description | Number in input list | Number in reference list | *p*-value |
|---|---|---|---|---|---|
| Upregulated genes (24 hpi) | | | | | |
| 10530 | Aminosugars metabolism | carbohydrate metabolism | 9 | 76 | 1.15E-03 |
| 10910 | Nitrogen metabolism | energy metabolism | 8 | 83 | 2.04E-03 |
| 10350 | Tyrosine metabolism | amino acid metabolism | 10 | 130 | 3.23E-03 |
| 10460 | Cyanoamino acid metabolism | other amino acids metabolism | 4 | 31 | 9.99E-03 |
| 10480 | Glutathione metabolism | other amino acids metabolism | 16 | 127 | 4.14E-07 |
| 10770 | Pantothenate and CoA biosynthesis | cofactors and vitamin metabolism | 5 | 39 | 4.16E-03 |
| 10940 | Phenylpropanoid biosynthesis | biosynthesis of secondary metabolites | 40 | 187 | 2.00E-12 |
| 11000 | Single reactions | other | 11 | 154 | 3.65E-03 |
| 34020 | Calcium signaling pathway | signal transduction | 9 | 128 | 9.05E-03 |
| 30008 | Ethylene signaling | hormone signaling | 15 | 232 | 2.00E-03 |
| 34626 | Plant-pathogen interaction | plant specific signaling | 25 | 311 | 1.75E-06 |
| 60003 | AP2/EREBP | transcription factor | 10 | 131 | 3.41E-03 |
| 60066 | WRKY | transcription factor | 19 | 62 | 1.93E-11 |
| 60069 | ZIM | transcription factor | 4 | 13 | 3.36E-04 |
| Downregulated genes (24 hpi) | | | | | |
| 10500 | Starch and sucrose metabolism | carbohydrate metabolism | 12 | 324 | 2.71E-04 |
| 44110 | Cell cycle | cell growth and death | 21 | 316 | 3.85E-11 |
| 44810 | Regulation of actin cytoskeleton | cell motility | 27 | 340 | 5.85E-16 |
| 60076 | Other GTF | | 2 | 6 | 1.79E-03 |
| Upregulated genes (96 hpi) | | | | | |
| 10640 | Propanoate metabolism | carbohydrate metabolism | 4 | 63 | 6.66E-04 |
| 50121 | Porters cat 1 to 6 | transporter catalogue | 6 | 160 | 5.20E-04 |
| Downregulated genes (96 hpi) | | | | | |
| 40006 | cell wall | cell growth and death | 12 | 445 | 1.31E-07 |

One of the earliest cellular responses following plant-pathogen interaction is the production of reactive oxygen species (ROS). Upon *B. cinerea* infection, genes putatively encoding enzymes involved in oxidative stress such as GST, ascorbate oxidase, 2OG-Fe(II) oxygenase, and cytochrome P450 monooxygenases were strongly upregulated (Table 2.2 and Supplemental Table S2.7). ROS accumulation at 24 hpi was proven by a localized green fluorescence emitted at the site of penetration in flowers obtained from a cytoplasmic HyPer (cHyPer) grapevine transgenic line expressing a cytosolic hydrogen peroxide molecular sensor (Figure 2.5).

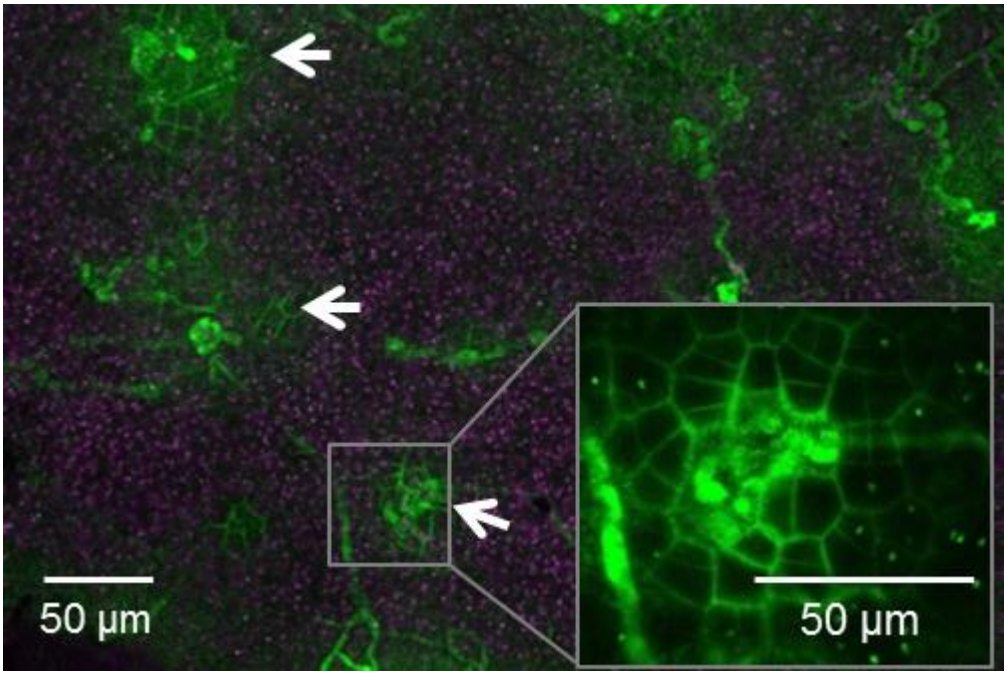



Figure 2.5. Confocal image of cytoplasmic HyPer grapevine transgenic flowers infected with *B. cinerea*. A higher intensity of HyPer fluorescence is evident 24 hpi at the penetration site of *B. cinerea*, compared with the rest of the plant tissue, indicating a localized and specific $H_2O_2$ accumulation (shown by arrows). The inset at higher magnification clearly shows that the bright signal comes from the cytosol of proximal cells to the site of infection.

Several genes encoding membrane-localized receptor-like kinases (*RLK*), such as, *Clavata1 receptor kinase*, *Wall-associated kinase 1*, and *Brassinosteroid insensitive 1– associated kinase 1* (*BAK1*), which have been characterized in connection with immune responses to necrotrophic pathogens (Kemmerling et al., 2007; Brutus et al., 2010), were also upregulated at 24 hpi (Supplemental Table S2.7). Genes associated with phytohormones, known to be involved in pathogen response signaling, were also differentially expressed (Figure 2.4 and Table 2.1).

Table 2.2. Selected differentially expressed grapevine genes due to *B. cinerea* inoculation (at 24 and 96 hpi)

| Id | Fold change (log2) 24 hpi | 96 hpi | Functional annotation |
|---|---|---|---|
| Recognition and signaling | | | |
| VIT_15s0046g02220 | 2.67 | | ACC synthase |
| VIT_07s0031g01070 | 2.21 | | Ascorbate oxidase |
| VIT_14s0030g02150 | 2.04 | 2.33 | Calmodulin |
| VIT_11s0016g03080 | 1.42 | | Clavata1 receptor kinase (CLV1) |
| VIT_12s0035g00610 | 6.01 | | CYP82M1v3 |
| VIT_18s0001g00030 | 1.01 | 2.79 | CYP87A2 |
| VIT_17s0000g07400 | 1.01 | | Disease resistance protein (EDS1) |
| VIT_17s0000g07420 | 1.38 | | Enhanced disease susceptibility 1 (EDS1) |
| VIT_02s0234g00130 | 1.60 | | Ethylene responsive element binding factor 1 |
| VIT_15s0048g01350 | 2.22 | | Gibberellin receptor GID1L3 |
| VIT_08s0040g00920 | 2.94 | 1.71 | Glutathione S-transferase 25 GSTU7 |
| VIT_11s0016g00710 | 0.83 | | Jasmonate ZIM-domain protein 1 |
| VIT_01s0011g03650 | 2.21 | | Map kinase substrate 1 MKS1 |
| VIT_00s0250g00090 | 4.42 | 2.72 | Oxidoreductase, 2OG-Fe(II) oxygenase |
| VIT_03s0063g02440 | | -1.71 | Proline extensin-like receptor kinase 1 |
| VIT_13s0064g01790 | -1.62 | | R protein MLA10 |
| VIT_00s0748g00020 | 4.14 | | Receptor kinase RK20-1 |
| VIT_17s0000g04400 | 1.38 | | Wall-associated kinase 1 (WAK1) |
| Trascription factors | | | |
| VIT_07s0005g03220 | | 3.53 | |
| VIT_11s0016g02070 | 3.09 | 1.43 | Basic helix-loop-helix (bHLH) family |
| VIT_07s0005g03340 | 1.87 | | Myb domain protein 14 |
| VIT_19s0027g00860 | 3.64 | | NAC domain-containing protein 42 |
| VIT_08s0058g00690 | 1.65 | | WRKY DNA-binding protein 33 |
| VIT_14s0068g01770 | 3.29 | | WRKY DNA-binding protein 75 |
| Cell wall | | | |
| VIT_14s0128g00970 | 2.75 | 1.40 | Germin-like protein 3 |
| VIT_05s0077g01280 | -1.72 | -2.40 | Glycosyl hydrolase family 3 beta xylosidase |
| VIT_06s0009g02560 | 3.21 | | Pectinesterase family |
| VIT_08s0007g08330 | -4.80 | | Polygalacturonase PG1 |
| VIT_09s0054g01080 | 3.10 | | Polygalacturonase QRT3 |
| VIT_06s0004g01990 | 4.87 | 3.15 | Proline-rich extensin-like family protein |
| VIT_03s0017g01990 | 1.79 | | UDP-glucose glucosyltransferase |

| Id | Fold change (log2) 24 hpi | 96 hpi | Functional annotation |
|---|---|---|---|
| Response to stress and secondary metabolism | | | |
| VIT_18s0001g04280 | 5.07 | | (-)-germacrene D synthase |
| VIT_11s0052g01110 | 1.96 | | 4-coumarate-CoA ligase 1 |
| VIT_04s0008g07250 | 2.04 | | Aspartyl protease |
| VIT_05s0077g01540 | 5.43 | | Bet v I allergen |
| VIT_16s0098g00850 | 0.68 | | Caffeic acid O-methyltransferase |
| VIT_16s0100g00860 | 4.99 | | Chalcone synthase |
| VIT_11s0149g00280 | 2.13 | | Chitinase A |
| VIT_03s0180g00250 | 4.41 | | Cinnamyl alcohol dehydrogenase |
| VIT_16s0039g02350 | 1.07 | | Dihydroflavonol 4-reductase |
| VIT_18s0122g01150 | 6.57 | 2.61 | Diphenol oxidase |
| VIT_06s0004g01020 | 5.62 | 2.10 | Dirigent protein |
| VIT_07s0031g01380 | 2.04 | 0.96 | ferulate 5-hydroxylase |
| VIT_05s0020g05000 | 1.70 | | Inhibitor of trypsin and hageman factor (CMTI-V) |
| VIT_18s0001g00850 | 6.48 | 2.84 | Laccase |
| VIT_16s0098g00460 | 3.29 | | Lipase class 3 |
| VIT_14s0083g00850 | | -1.67 | Lipase GDSL 7 |
| VIT_13s0067g00050 | 3.32 | | Myrcene synthase |
| VIT_15s0048g02430 | 1.65 | | Naringenin,2-oxoglutarate 3-dioxygenase |
| VIT_05s0077g01530 | 4.94 | 1.56 | Pathogenesis protein 10 |
| VIT_05s0077g01550 | 4.62 | | Pathogenesis protein 10.3 |
| VIT_03s0088g00750 | 1.45 | | Pathogenesis related protein 1 precursor |
| VIT_01s0010g02020 | 7.12 | 2.09 | Peroxidase |
| VIT_16s0039g01280 | 5.40 | | Phenylalanin ammonia-lyase |
| VIT_00s2849g00010 | 5.83 | | Phenylalanine ammonia-lyase |
| VIT_02s0025g02920 | 1.67 | | Quercetin 3-O-methyltransferase 1 |
| VIT_08s0058g00790 | 1.51 | | Secoisolariciresinol dehydrogenase |
| VIT_16s0100g01010 | 4.64 | | Stilbene synthase (VvSTS29) |
| VIT_16s0100g01130 | 4.34 | | Stilbene synthase (VvSTS41) |
| VIT_16s0100g01160 | 4.39 | | Stilbene synthase (VvSTS45) |
| VIT_16s0100g00990 | 4.60 | | Stilbene synthase 2 (VvSTS27) |
| VIT_16s0100g00950 | 4.63 | | Stilbene synthase 3 (VvSTS25) |
| VIT_02s0025g04230 | 2.21 | | Thaumatin |
| VIT_11s0065g00350 | 3.54 | | Trans-cinnamate 4-monooxygenase |

According to the number of DE genes related to ET biosynthesis or signaling, this hormone seems to be important in the interaction between grapevine flower and *B. cinerea*. Two ACC synthase and one ACC oxidase genes in addition to seven ET responsive TFs were differentially expressed (Supplemental Table S2.7). Also genes encoding an SA marker *PR1* and the plant defense regulator involving SA signaling *Enhanced disease susceptibility 1* (Wiermer et al., 2005) were upregulated in the infected sample at 24 hpi (Table 2.2). Jasmonate ZIM-domain protein, a marker for JA, was slightly but significantly upregulated. Other genes involved in the synthesis of and signalling by phytohormones (apart from ET, JA and SA pathways) showed changes in transcript levels following inoculation with *B. cinerea*, suggesting a complex hormonal interplay. For example, five genes encoding nitrilase and nitrile hydratases, putatively involve in indole 3-acetic acid synthesis, were differentially regulated, as well as receptor genes for gibberellic acid (GA) and abscisic acid (ABA) (Supplemetal Data S2). The global hormonal alterations related to infection were evaluated by Hormonometer software (Volodarsky et al., 2009) and presented in Supplemental Figure S2.8. The results of this analysis also suggested the involvement of several hormones.

Following the *Botrytis*-induced signaling cascades, about 100 TFs were differentially expressed in infected flowers. Most prominent were genes encoding WRKY TFs, Myb domain proteins, ethylene-responsive element-binding proteins, and NAC TFs (Supplemental Table S2.7). Among the transcriptional regulators previously associated to the defense reaction were *WRKY33*, a key transcriptional regulator involved in defense against *B. cinerea* and *Plasmopara viticola* in *Arabidopsis* and grapevine, respectively (Birkenbihl et al., 2012; Merz et al., 2015), and *Myb14*, which in grapevine regulates stilbene biosynthesis (Höll et al., 2013). This fast and strong induction of specific TFs leads to the activation of specific pathways, clearly related to plant defense. A number of genes encoding different classes of PR-proteins, such as chitinase, and Bet v I allergen, and Beta 1-3 glucanase were upregulated, up to 40 fold, following *Botrytis* inoculation. In this work, the transcription levels of *VvPR10.1* and *VvPR10.3*, and their putative regulator *WRKY33*, as indicated by previous studies for *VvPR10.1* (Dadakova et al., 2015; Merz et al., 2015), were analyzed in more detail. The transcription profiles measured by qPCR at five time points within the first 96hpi revealed that the transcript level 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 PR-proteins were always higher than control, except

*VvPR10.1* at 48 hpi (Figure 2.6). *VvPR10.1* reached its peak level at 24 hpi, dropped at 48 hpi and then slowly increased at 72 and 96 hpi; whereas *VvPR10.3* showed a double peak of expression, the first at 24 hpi and a second one at 96 hpi. Proteases including those involved in defense such as subtilisin-like protease, aspartic protease, and serine protease inhibitor were also more expressed in *Botryits*-inoculated than in mock-treated flowers (Supplemental Table S2.7).

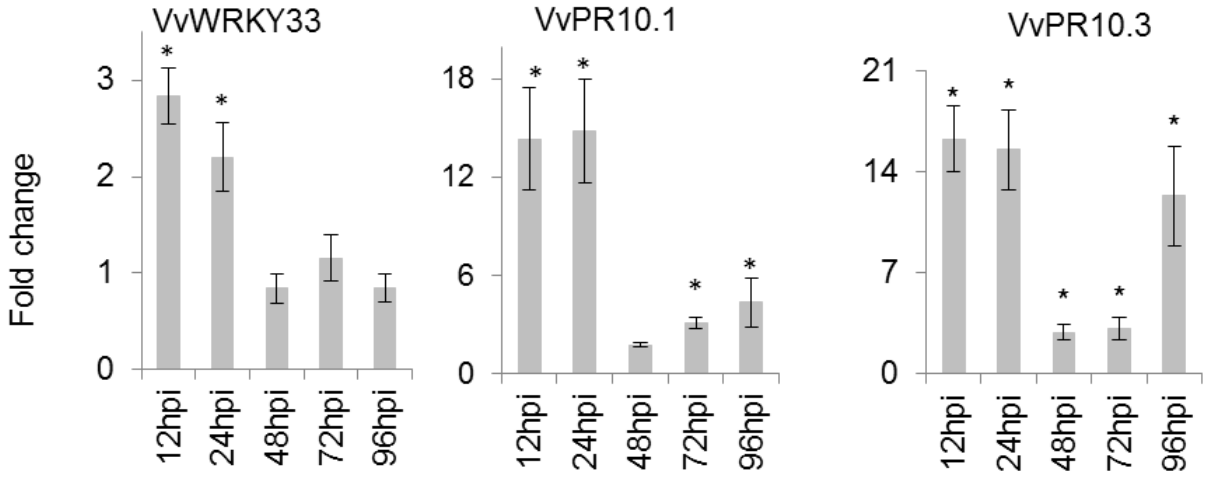



Figure 2.6. Expression profiles of *VvWRKY33*, *VvPR10.1*, and *VvPR10.3* following *B. cinerea* inoculation. Gene expression levels were determined by qPCR. Bars represent fold change of inoculated sample relative to mock-inoculated sample at each post-inoculation time. Normalization based on the expression levels of actin, *VvACT* and tubulin, *VvTUB* was carried out before calculating fold changes. Error bar represents standard error of the mean of three biological replicates. Asterisks (*) indicate statistically significant difference ($P < 0.05$) between mock- and *B. cinerea*- inoculated samples within a post-inoculation time using unpaired heteroscedastic Student's t test. hpi, hours post inoculation.

### 2.4.4. Secondary metabolism, mainly related to polyphenols, is upregulated in infected flowers

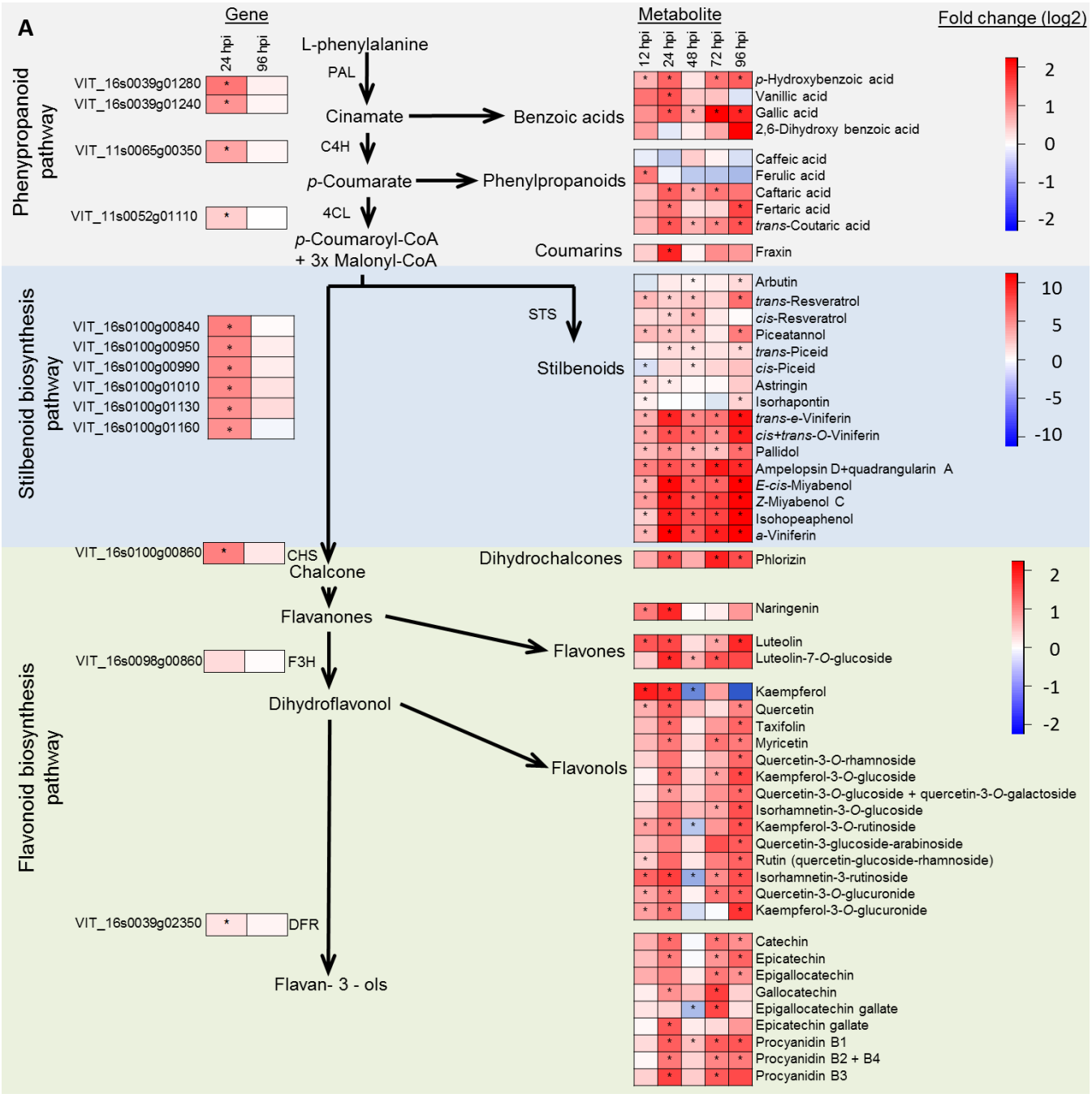
The RNA-Seq results underlined a reprogramming in the host transcriptome towards secondary metabolism, especially at 24 hpi (Figure 2.4 and Supplemental Table S2.7). Several genes related to terpenoid, benzoic acids, monolignol precursors, stilbenoid and flavonoid biosynthesis were strongly upregulated at the initial stage of *B. cinerea* infection. From the enrichment analysis, secondary metabolic process, protein modification process and phenylpropanoid biosynthesis were among the enriched functional categories at 24 hpi (Table 2.1 and Supplemental Table S2.5). To confirm these RNA-Seq observations, targeted secondary metabolites, mainly polyphenols, were quantified by UHPLC-DAD-MS at five time points between 12 and 96 hpi. The analysis revealed that a number of benzoic acid, monolignol precursors, stilbenoid

compounds and flavonoids, and their derivatives, many of which are associated with plant defense, were detected at higher concentrations in *Botrytis*-infected flowers as compared to mock-treated flowers (Supplemental Table S2.8), suggesting a huge defense-oriented metabolome reprogramming following *B. cinerea* inoculation. Figure 2.7 shows a heat map of the concentrations of metabolites in correlation with gene expression profiles, taken from the RNA-Seq result. In the phenylpropanoid pathway, ten genes encoding PAL were upregulated, between 13 and 55 fold at 24 hpi (Supplemental Table S2.7). Furthermore, genes encoding key enzymes in the pathway cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL) had a fold change of about 12 and 4 times, respectively, also at 24 hpi (Table 2.2). The concentrations of benzoic acids (*p*-hydroxybenzoic acid, vanillic acid, and gallic acid) and monolignol precursors (ferulic acid, caftaric acid, fertaric acid, and *trans*-coutaric acid) were higher in infected flowers, at least in one of the post inoculation time points considered (Figure 2.7A).

Regarding stilbenoids biosynthesis, an overwhelming number of STS genes were highly upregulated at 24 hpi (Supplemental Table S2.7). Of the 46 *V. vinifera* genes functionally annotated as STS, more than 80% of them were expressed in the infected flowers with a relative induction between 15 and 90 fold. Two genes which encode VvMYB14, an R2R3-MYB–type TF regulating stilbene biosynthesis (Höll et al., 2013), were also upregulated at 24 hpi (Table 2.2), suggesting that this regulatory circuit is activated at 24 hpi. The expression profiles of *VvMYB14* and two STS genes, *VvSTS29* and *VvSTS41* (Vannozzi et al., 2012) were further monitored using qPCR (Figure 2.7B). Because of high sequence similarity among STS genes (Vannozzi et al., 2012), the primers used for *VvSTS29* also detect the isoforms *VvSTS25* and *VvSTS27*, while the primers used for *VvSTS41* detect the isoform *VvSTS45* too (Table 2.2) (Höll et al., 2013). These results showed a strong coinduction between the STS genes and *VvMYB14* in grapevine flowers/fruitlets in response to *B. cinerea* infection, confirming the results of the RNA-Seq analysis (Figure 2.7B). The expression patterns observed in the qPCR assay also fitted with the quantification of stilbenoids. The phytoalexin resveratrol and its monomeric-derivatives piceatannol and *trans*-piceid were detected at higher concentrations in the infected flowers/fruitlets than control, in most of the post inoculation time points examined (Figure 2.7A). The other monomers astringin and isorhapontin, both tetrahydroxystilbenes with antifungal activity (Hammerbacher et al., 2011), were also induced. All of the quantified oligomeric resveratrols (dimers: *trans*-ε-viniferin, *cis*+*trans*-ο-viniferin, pallidol, ampelopsin D and

quadrangularin A, and E-*cis*-miyabenol; trimers: Z-miyabenol C and α-viniferin; and the tetramer isohopeaphenol) were found highly concentrated in the infected flowers/fruitlets as compared to the control throughout the post inoculation time points examined (Figure 2.7A). The quantities of the stress related *trans*-ε- and α-viniferins, which are also involved in grapevine – *B. cinerea* interaction (Langcake, 1981), ranged from 0.9 to 13.4 µg/g fresh weight (fw) and 2.8 to 151.8 µg/g fw, respectively, in the inoculated fruitlets as compared to basal levels in mock inoculated controls. A similar increase in concentration was also observed in Z-miyabenol C and isohopeaphenol: 1.2 to 46.4 µg/g fw and 0.2 to 20.9µg/g fw, respectively (Supplemental Table S2.8). Such an increase in the concentration of these monomeric and oligomeric stilbenoids suggests that they contribute to inhibiting the pathogen's advancement in colonizing the fruitlet.

In addition to STS genes, chalcone synthase (CHS), and dihydroflavonol-4-reductase (DFR), key flavonoid biosynthetic genes, were differentially expressed at 24 hpi (Table 2.2). The quantification of flavonoids revealed that flavanones, flavones, flavonols, and flavan-3-ols were detected at higher concentrations following *Botrytis* inoculation, most pronouncedly at 24, 72 and 96 hpi (Figure 2.7A). A number of flavonoids known to restrict fungal growth and in some cases also inhibit stilbene oxidases (Goetz et al., 1999; Guestsky et al., 2005; Puhl and Treutter, 2008; Nagpala et al., 2016), such as naringenin, kaempferol, quercetin, taxifolin, quercetin-3-glucuronide, catechin, epicatechin, epicatechin gallate, and procyanidin B1, B2, B3 and B4, were elevated within 24 hpi and often after 72hpi compared to control samples (Figure 2.7A).


A
Gene
24 hpi
96 hpi
Metabolite
12 hpi
24 hpi
48 hpi
72 hpi
96 hpi
Fold change (log2)
Phenypropanoid pathway
L-phenylalanine
PAL
Cinamate
Benzoic acids
C4H
p-Coumarate
Phenylpropanoids
4CL
p-Coumaroyl-CoA + 3x Malonyl-CoA
Coumarins
VIT_16s0039g01280
VIT_16s0039g01240
VIT_11s0065g00350
VIT_11s0052g01110
p-Hydroxybenzoic acid
Vanillic acid
Gallic acid
2,6-Dihydroxy benzoic acid
Caffeic acid
Ferulic acid
Caftaric acid
Fertaric acid
trans-Coutaric acid
Fraxin
Stilbenoid biosynthesis pathway
STS
Stilbenoids
VIT_16s0100g00840
VIT_16s0100g00950
VIT_16s0100g00990
VIT_16s0100g01010
VIT_16s0100g01130
VIT_16s0100g01160
Arbutin
trans-Resveratrol
cis-Resveratrol
Piceatannol
trans-Piceid
cis-Piceid
Astringin
Isorhapontin
trans-ε-Viniferin
cis+trans-O-Viniferin
Pallidol
Ampelopsin D+quadrangularin A
E-cis-Miyabenol
Z-Miyabenol C
Isohopeaphenol
α-Viniferin
Flavonoid biosynthesis pathway
VIT_16s0100g00860
CHS
Chalcone
Dihydrochalcones
Phlorizin
Flavanones
Naringenin
Flavones
VIT_16s0098g00860
F3H
Luteolin
Luteolin-7-O-glucoside
Dihydroflavonol
Flavonols
Kaempferol
Quercetin
Taxifolin
Myricetin
Quercetin-3-O-rhamnoside
Kaempferol-3-O-glucoside
Quercetin-3-O-glucoside + quercetin-3-O-galactoside
Isorhamnetin-3-O-glucoside
Kaempferol-3-O-rutinoside
Quercetin-3-glucoside-arabinoside
Rutin (quercetin-glucoside-rhamnoside)
Isorhamnetin-3-rutinoside
Quercetin-3-O-glucuronide
Kaempferol-3-O-glucuronide
VIT_16s0039g02350
DFR
Flavan- 3 - ols
Catechin
Epicatechin
Epigallocatechin
Gallocatechin
Epigallocatechin gallate
Epicatechin gallate
Procyanidin B1
Procyanidin B2 + B4
Procyanidin B3

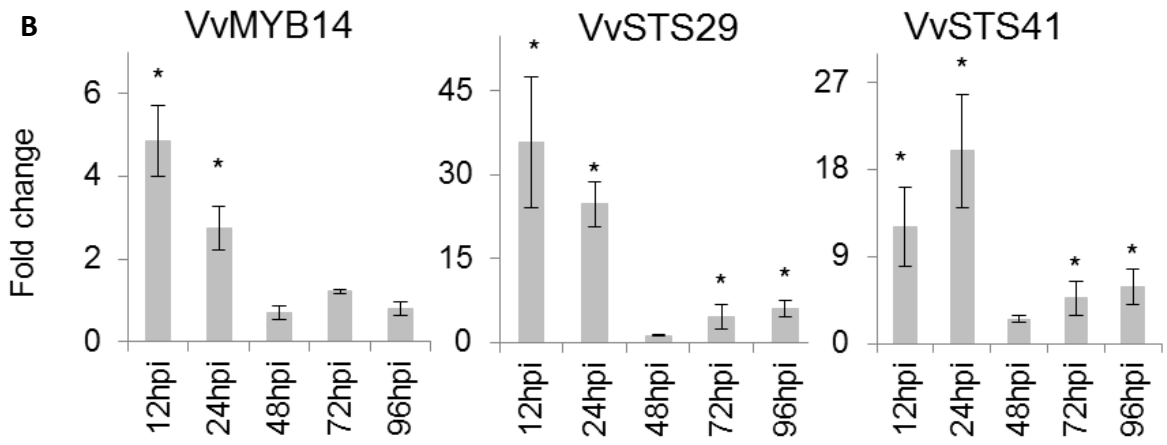



Figure 2.7. Transcript and metabolite analyses of the grapevine phenylpropanoid pathway upon *B. cinerea* inoculation. A, Heatmaps of gene expression (from RNA-Seq result) and secondary metabolite concentration (µg/g fw, from HPLC-DAAD-MS) expressed as fold change. Fold changes were computed based on the ratio of average values in *B. cinerea*- and mock-inoculated flowers, for each time point. CHS, chalcone synthase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; DFR, dihydroflavonol-4-reductase; F3H, flavanone 3-hydroxylase; PAL, phenylalanine ammonia lyase; STS, stilbene synthase (out of 39, only 6 are dipcted). B, Expression profile of *VvSTS29* (*-27-25*), *VvSTS41* (*-45*), and *VvMYB14*. Gene expression levels were determined by qPCR. Bars represent fold change of inoculated sample relative to mock-inoculated sample at each post-inoculation time. Normalization based on the expression levels of actin, *VvACT* and tubulin, *VvTUB* was carried out before calculating fold changes. Error bar represents standard error of the mean of three biological replicates. Asterisks (*) indicate statistically significant difference ($P < 0.05$) between mock- and *B. cinerea*-inoculated samples within a post-inoculation time using unpaired heteroscedastic Student's t test. hpi, hours post inoculation.

### 2.4.5. Infection triggers cell wall reinforcement

Reinforcing the cell wall to combat pathogen intrusion is a well-established mechanism in plants. A sign of cell wall apposition (CWA) at the site of penetration was observed by the autofluorescence of CWA regions (Figure 2.8A). The GO enrichment analyses also proposed that the L-phenylalanine catabolic process, cell wall, and extracellular region were among the enriched functional classes (Supplemental Table S2.5). This preliminary evidence was strengthened by modulation of genes encoding cell wall modifying enzymes such as pectinesterases (PEs), catalyzing the hydrolysis of methylester groups from polygalacturonans, polygalacturonases (PGs) and pectate lyases, degrading the pectic homogalacturonans (Supplemental Table S2.7). PE is involved both in cell wall loosening, by making polygalacturonans accessible to degradation by PG, and cell wall strengthening, by increasing the availability of polygalacturonan to $Ca^{2+}$ binding (Micheli, 2001). The induction and suppression

of PEs and PGs observed in the RNA-Seq result may suggest that the flower is fine tuning between cell wall stiffening, as a barrier against the pathogen, and cell expansion and separation, as the flower itself is also in its active growth phase. Complementary to this, we observed that genes encoding germin-like protein 3 (GLP3) and proline-rich extensin-like protein (EXT), proteins involved in $H_2O_2$-mediated oxidative cross-linking to toughen cell walls during pathogen attack (Bradley et al., 1992; Godfrey et al., 2007; Kelloniemi et al., 2015), were highly upregulated both at 24 and 96 hpi (Table 2.2). Grapevine genes which encode enzymes involved in monolignol biosynthesis, cinnamyl alcohol dehydrogenase and *trans*-cinnamate 4-monooxygenase, were also highly upregulated (Table 2.2). These all suggest that cell wall fortification process might have been activated due to the infection.

Cell wall reinforcement is one of the possible mechanisms by which the grapevine flower arrests the advancement of *Botrytis*. Therefore, ten genes encoding enzymes in the monolignol biosynthesis pathway (based on the VitisNet annotations of phenylpropanoid biosynthesis), from the differentially expressed genes of RNA-Seq analysis, were selected for further investigation with a qPCR assay. The quantities of L-phenylalanine and other seven intermediate metabolites in this pathway were also measured by HPLC-DAD-MS (Supplemental Table S2.8). Transcripts of cinnamate 4-hydroxylase (*VvC4H*) and 4-coumarate-CoA ligase (*Vv4CL*), enzymes in the upstream of the pathway, were upregulated at the onset of *B. cinerea* infection, between 12 and 24 hpi, with *VvC4H* showing a peak at 12 hpi (Figure 2.8B). A similar trend was observed for cinnamoyl CoA reductase (*VvCCR*), the first enzyme specific to monolignol synthesis (Naoumkina et al., 2010). Caffeic acid o-methyltransferase (*VvCOMT*) and caffeoyl-CoA O-methyltransferase (*VvCCoAMT*) were slightly, but significantly, upregulated at 24 hpi only; however, ferulate 5-hydroxylate (*VvF5H*) was upregulated throughout the post inoculation time points examined. Cinnamyl alcohol dehydrogenase (*VvCAD*) was significantly upregulated up to 48 hpi. *VvCAD* is the final enzyme in the sequential actions of *Vv4CL*, *VvCCR*, and *VvCAD* to reduce aldehyde derivatives into the corresponding alcohols before monolignols synthesis. In the cell wall, monolignols undergo oxidative polymerization, catalyzed by peroxidases/laccases (Naoumkina et al., 2010). A strong upregulation of a lignin-forming anionic peroxidase-like (*VvPER*) was observed throughout the post inoculation time points examined, with the highest induction being within 24 hpi. With regard to laccase, about 10 putative grapevine laccase genes, having the same KEGG enzyme code of *Arabidopsis thaliana* lignin laccase (Zhao et al., 2013),

were also found extremely upregulated, up to 90 fold (Supplemental Table S2.7).

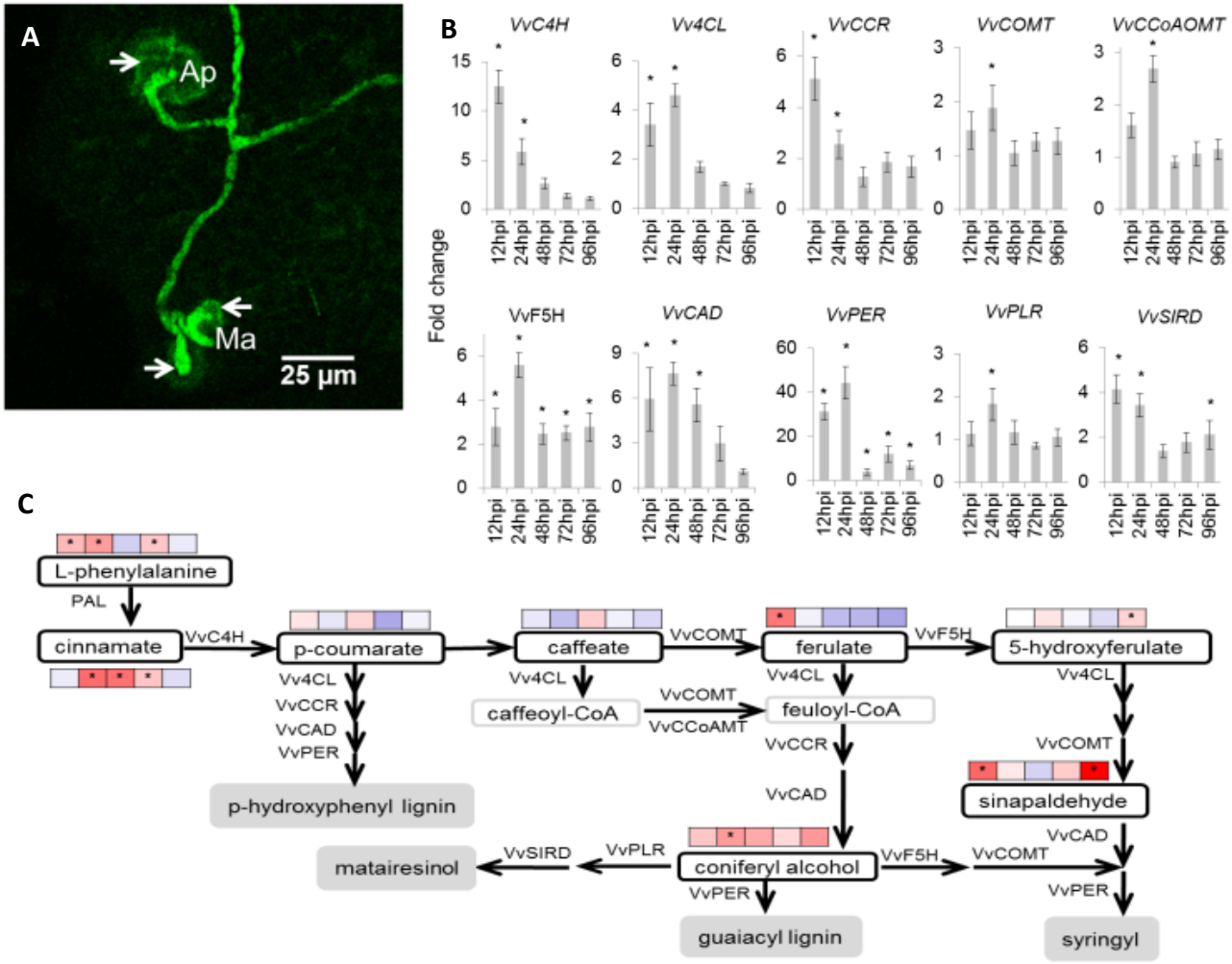



Figure 2.8. Cell wall apposition and monolignol biosynthesis pathway in *B. cinerea* infected flowers. A, Confocal microscope image at 30 hpi of GFP-labeled *B. cinerea*. Autofluorescence around penetration site (shown by arrow) indicates cell wall apposition. Ap, appressoria; and Ma, multicellular appresoria (infection cushions). B, Expression profile of genes encoding key enzymes of the grapevine monolignol biosynthetic pathway. *VvCAD*, cinnamyl alcohol dehydrogenase; *VvCOMT*, caffeic acid o-methyltransferase; *VvCCoAMT*, caffeoyl-CoA O-methyltransferase; *VvC4H*, cinnamate 4-hydroxylase; *VvCCR*, cinnamoyl CoA reductase; *Vv4CL*, 4-coumarate-CoA ligase; *VvF5H*, ferulate 5-hydroxylate; *PAL*, phenylalanine ammonia lyase; *VvPER*, peroxidase (lignin-forming anionic peroxidase-like); *VvPLR*, pinoresinol/lariciresinol reductase; *VvSIRD*, secoisolariciresinol dehydrogenase. Gene expression levels were determined by qPCR. Bars represent fold change of *B. cinerea*-inoculated sample relative to mock-inoculated sample at each post-inoculation time. Normalization based on the expression levels of actin, *VvACT* and tubulin, *VvTUB* was carried out before calculating fold changes. Error bar represents standard error of the mean of three biological replicates. C, Heatmap of monolignol precursors superimposed to the biosynthetic pathway. The amounts of monolignol precursors (µg/g fw) were quantified by HPLC-DAAD-MS. Fold changes were computed based on the ratio of average values of *B. cinerea*- and mock-inoculated flowers, for each time point. Monolignol and lignan compounds are highlighted in gray background. Asterisks (*) indicate statistically significant difference ($P < 0.05$) between mock- and *B. cinerea*-inoculated samples within a post-inoculation time using unpaired heteroscedastic Student's t test.

Pinoresinol/lariciresinol reductase (*VvPLR*) and secoisolariciresinol dehydrogenase (*VvSIRD*), which catalyze subsequent metabolic steps to give rise to matairesinol, a lignan, as well as most of the DE genes which putatively encode dirigent-like proteins, a disease resistance-responsive family protein involved in lignan biosynthesis, were also upregulated in response to *B. cinerea*.

The quantification of metabolites also revealed that the concentrations of L-phenylalanine and cinnamate, which represent the two key entry substrates in the monolignol biosynthesis pathway, were significantly higher in *Botrytis*-inoculated flowers (Figure 2.8C). In contrast, the concentration of p-coumarate, caffeate, ferulate, and 5-hydroxyferulate were not different between *Botrytis*-inoculated flowers and control samples, probably due to their rapid conversion into the next metabolite of the pathway. Exceptions were the two intermediates, sinapaldehyde and coniferyl alcohol, metabolites found towards the end of the pathway, which were generally higher in the *Botryits*-inoculated flowers than in the control samples.

These results indicate that upon *B. cinerea* infection, cell wall fortification was among the defense mechanisms employed by the flowers/fruitlets to contain the pathogen in its quiescent state.

### 2.4.6. *Botrytis cinerea* transcripts expressed *in planta* during grapevine flower infection

The signal of *B. cinerea* transcripts detected in inoculated flowers was very low (Supplemental Table S2.2). As mentioned above, the reasons could be the limited amount of conidia used for inoculation, about 300 conidia per flower, and/or the arrest in fungal growth after penetration resulting in a very low abundance of fungal RNA in the samples. Therefore, it was not possible to perform a statistical comparison between the transcriptome of the infecting pathogen vs. the PDB-grown fungus in order to identify the pathogenicity genes of *Botrytis* and an alternative approach was applied. Genes from *B. cinerea* were considered expressed if they were represented by an average of at least 10 reads in the three biological replicates of inoculated flower samples. 1325 genes met these conditions and will be herein referred to as "*in planta* expressed genes". Of these, 751 and 59 were expressed only at 24 and 96 hpi, respectively; and 515 genes were expressed at both 24 and 96 hpi (Supplemental Table S2.9).

The set of *in planta* expressed genes were functionally annotated using Blast2GO (Conesa et al., 2005) and Amselem et al. (2011) (Supplemental Table S2.9). The joined biological meaning of the genes was visualized using the Combined Graph Function of Blast2GO based on their GO

slim terms, and primary metabolic process, nitrogen compound metabolic process, ion binding, oxidoreductase activity, and cytoplasmic component were among the most represented GO terms (Supplemental Table S2.10). This automatic annotation was manually curated to improve gene description and the most important functional categories are reported in Table 2.3. *In planta* expressed genes encompassed genes involved in pathogenesis, such as Carbohydrate-Active enZymes (CAZymes) devoted to plant cell wall degradation, in ROS production and detoxification, in toxins biosynthesis, as well as in transcriptional regulation; all these genes were more abundant at 24 hpi. Besides, many ribosomal and histone genes were equally abundant at 24 and 96 hpi indicating the maintenance of a basal metabolism during quiescence.

Table 2.3. Specific functions of *in planta* detected *B. cinerea* transcripts

| Functions of of *Botrytis cinerea* genes | No.of genes involved | |
|---|---|---|
| | 24hpi | 96hpi |
| Proteins identified as early secretome, within 16 h of germination (Espino et al., 2010) | 39 | 9 |
| Carbohydrate-Active Enzymes (CAZymes) (Amselem et al., 2011; Blanco-Ulate et al., 2014) | 203 | 64 |
| CAZymes acting on fungal cell wall | 36 | 16 |
| CAZymes acting on Plant Cell Wall | 56 | 11 |
| CAZymes acting on cellulose | 5 | 2 |
| CAZymes acting on hemicellulose | 20 | 3 |
| CAZymes acting on hemicellulose and pectin side chains | 9 | 0 |
| CAZymes acting on pectin | 23 | 1 |
| Proteins generating Reactive Oxygen Species (ROS) (Schumacher et al., 2014) | 10 | 2 |
| Proteins involved in the detoxification of ROS (Schumacher et al., 2014) | 23 | 8 |
| Protease (Amselem et al., 2011) | 38 | 8 |
| secondary metabolism key enzymes (Amselem et al., 2011) | 3 | 0 |
| 60S & 40S ribosomal protein (Amselem et al., 2011) | 78 | 78 |
| Appressorium-associated genes (orthologs in *Magnaporthe oryzae*) (Amselem et al., 2011) | 7 | 2 |
| Transporters | 64 | 22 |
| Transcription factors | 29 | 13 |
| Histone | 8 | 7 |
| Actin | 11 | 6 |

### 2.4.7. *Botrytis cinerea* genes required for pathogenesis are upregulated during flower infection

A group of 23 *in planta* expressed genes known to be related or possibly associated to *Botrytis* growth or pathogenesis were further characterized by qPCR to study their expression profiles during the early interaction with the grapevine flower, in comparison to their expression during

*Botrytis* growth in PDB medium. In addition, other two genes, glutathione S-transferase (*BcGST1*) and polygalacturonase2 (*BcPG2*), reported to be induced during pathogenesis were analyzed. qPCR expression profiles are shown in Figure 2.9, while gene names together with the expression level from the RNA-seq analysis are reported in Table 2.4.

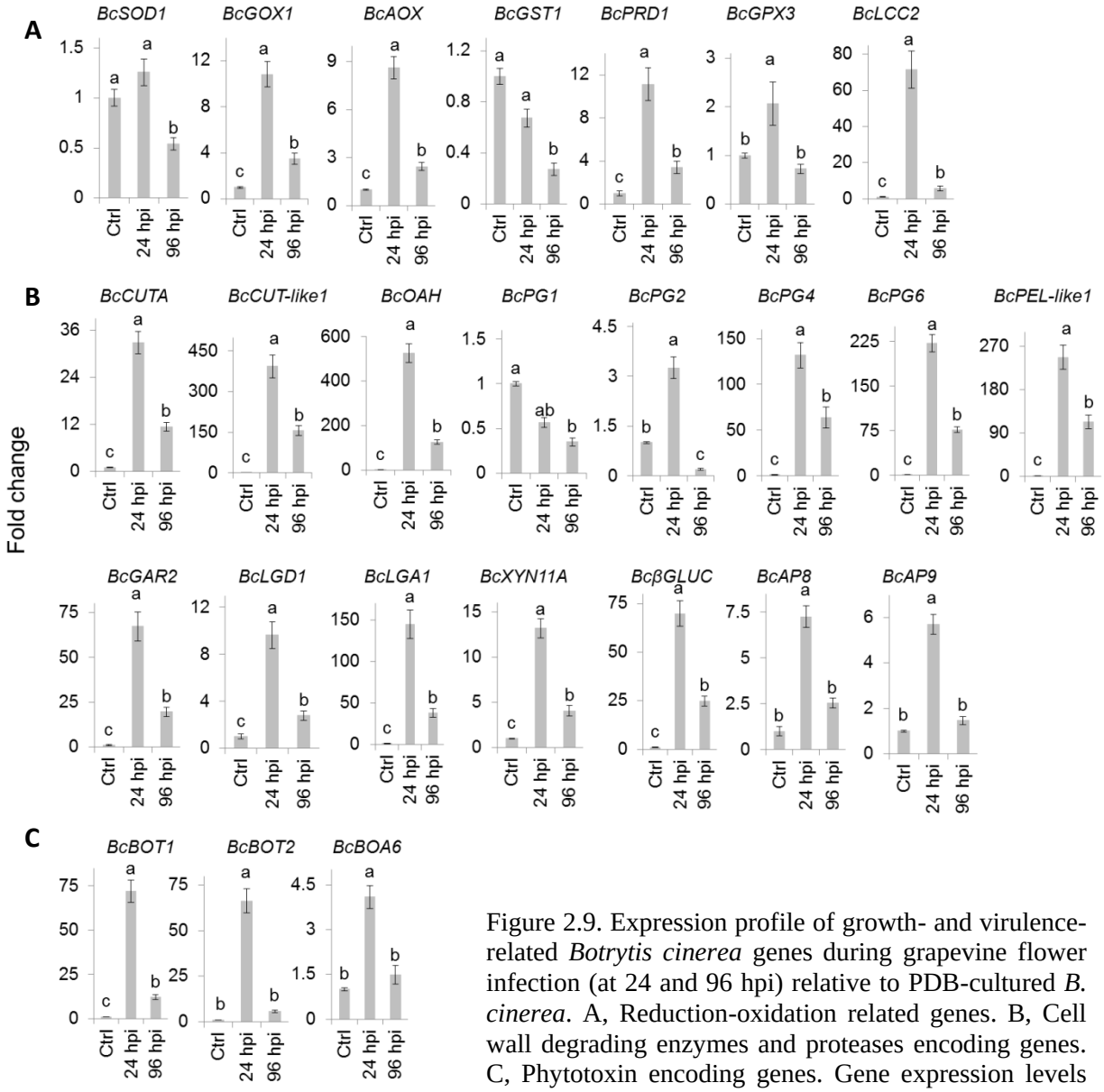



Figure 2.9. Expression profile of growth- and virulence-related *Botrytis cinerea* genes during grapevine flower infection (at 24 and 96 hpi) relative to PDB-cultured *B. cinerea*. A, Reduction-oxidation related genes. B, Cell wall degrading enzymes and proteases encoding genes. C, Phytotoxin encoding genes. Gene expression levels were determined by qPCR. Bars represent fold change of sample at 24 or 96 hpi relative to the PDB-cultured *B. cinerea* (Ctrl). Normalization based on the expression levels of ribosomal protein L5, *BcRPL5*, and α tubulin, *BcTUBA*, was carried out before calculating fold changes. Error bar represents standard error of the mean of three biological replicates. Expression values followed by a common letter are significantly not different between samples, according to Tukey's Honestly Significant Difference test ($P \leq 0.05$), using one way ANOVA of log2(NRQ).

Table 4. RNA-seq reads of *B. cinerea* transcripts checked by qPCR assay

| | | | Average no. of raw reads from RNA-seq analysis | | |
|---|---|---|---|---|---|
| | | | In planta expressed | | PDB culture |
| Abbreviation | Transcript description | Gene ID | 24hpi | 96hpi | |
| *BcSOD1* | Superoxide dismutase1 | Bcin03g03390 | 76 | 26 | 9,411[a] |
| *BcGOX1* | Galactose oxidase | Bcin13g05710 | 21 | | 609 |
| *BcAOX* | Alcohol oxidase | Bcin07g03040 | 24 | | 490 |
| *BcGST1* | Glutathione S-transferase | Bcin10g00740 | | | 2,655[a] |
| *BcPRD1* | Dyp-type peroxidase | Bcin13g05720 | 19 | | 312 |
| *BcGPX3* | Glutathione peroxidase | Bcin03g01480 | 23 | | 2,871[a] |
| *BcLCC2* | Laccase2 | Bcin14g02510 | 15 | | 32[b] |
| *BcCUTA* | Cutinase | Bcin15g03080 | 15 | | 54[b] |
| *BcCUT-like1* | Cutinase | Bcin01g09430 | 68 | 11 | 9[b] |
| *BcOAH* | Oxaloacetate acetylhydrolase | Bcin12g01020 | 386 | | 38[b] |
| *BcPG1* | Polygalacturonase1 | Bcin14g00850 | 209 | 175 | 147,821[a] |
| *BcPG2* | Polygalacturonase2 | Bcin14g00610 | | | 362 |
| *BcPG4* | Polygalacturonase4 | Bcin03g01680 | 44 | | 47[b] |
| *BcPG6* | Polygalacturonase6 | Bcin02g05860 | 75 | | 62 |
| *BcPEL-like1* | Pectate lyase | Bcin03g05820 | 87 | 24 | 40[b] |
| *BcGAR2* | D-galacturonic acid reductase2 | Bcin03g01500 | 37 | | 104 |
| *BcLGD1* | D-galactonate dehydrogenase | Bcin01g09450 | 61 | 10 | 804 |
| *BcLGA1* | 2-keto-3-deoxy-L-galactonate aldolase | Bcin03g01490 | 70 | 13 | 66 |
| *BcXYN11A* | Endo-beta-1,4-xylanase | Bcin03g00480 | 18 | | 129 |
| *BcβGLUC* | Beta-glucosidase 1 precursor | Bcin10g05590 | 32 | | 75 |
| *BcAP8* | Aspartic proteinase8 | Bcin12g02040 | 30 | 12 | 1,064[a] |
| *BcAP9* | Aspartic proteinase9 | Bcin12g00180 | 16 | | 569 |
| *BcBOT1* | Botrydial biosynthesis1 | Bcin12g06380 | 58 | | 86 |
| *BcBOT2* | Botrydial biosynthesis2 | Bcin12g06390 | 41 | | 55 |
| *BcBOA6* | Botcinic acid6 | Bcin01g00060 | 13 | | 1,048[a] |

(a) transcripts whose average raw reads is in the top 25 %, most expressed, while (b) are those transcripts whose average reads is in the bottom 25 %, least expressed, in PDB culture.

All genes showed a sharp peak of expression at 24 hpi, except superoxide dismutase1 (*BcSOD1*), *BcGST1*, and the constitutively expressed *BcPG1*. Interestingly, the expression level at 24 hpi of some genes was extremely high, due to their very low level of expression in PDB culture, which was taken as a reference. These genes, which included oxaloacetate acetyl hydrolase (*BcOAH*), cutinase (*BcCUT-like1*), and pectate lyase (*BcPEL-like1*), appeared to be expressed exclusively during *Botrytis* attack, while other genes were also expressed in the absence of the host, but relatively much more during the host-pathogen interaction.

*B. cinerea* cutinases *BcCUTA* and *BcCUT*-like1are involved in the breaching of the cuticle layer by appressoria. This invasive step normally causes oxidative burst in the host (Schouten et al., 2002a) which is counteracted by the activation of scavenging mechanisms in the pathogen. The transcription levels of several *B. cinerea* genes taking part in the ROS-mediated fungus-plant

interaction were quantified (Figure 2.9A). *BcSOD1*, which plays role in oxidative stress response during cuticle penetration (Rolke et al., 2004); $H_2O_2$ generators galactose oxidase (*BcGOX1*) and alcohol oxidase (*BcAOX*); ROS scavengers *BcGST*, peroxidase1 (*BcPRD1*), and glutathione peroxidase (*BcGPX3*) (Schumacher et al., 2014); and *BcLCC2*, a gene involved in the oxidation of resveratrol and tannins (Schouten et al., 2002b), were all involved in the *Botrytis*-grapevine interaction. Necrotrophic fungi seem to stimulate the oxidative burst response of the plant and even contribute to it to favor the colonization process (review Heller and Tudzinsky, 2011).

A similar expression profile, albeit quantitavely different, was shown by cell wall degrading enzymes (CWDEs) (Figure 2.9B). *PG*, pectate lyase (*BcPEL-like1*), and oxaloacetate acetyl hydrolase (*BcOAH*), a gene with numerous functions including enhancing the activity of PG, are involved in pectin degradation. Galacturonate reductase (*BcGAR2*), galactonate dehydratase (*BcLGD1*), and 2-keto-3-deoxy-L- galactonate aldolase (*BcLGA1*), genes which play a role in the catabolic pathway of D-galacturonic acid (Zhang et al., 2011), a major component of pectin polysaccharides (Mohnen, 2008; Caffall and Mohnen, 2009), also had similar trends of expression. D-galacturonic acid may serve as an energy source when *B. cinerea* grows in and through plant cell walls (Zhang et al., 2011). The strong and similar expression pattern in pectin and D-galacturonic acid degrading enzymes suggested that the degradation of the pectin backbone was initiated at 24 hpi. The involvement of other CWDEs, such as the endo-beta-1,4-xylanase precursor (*BcXYN11A*), which degrades hemicellulose and also induces necrosis (Noda et al., 2010), beta-glucosidase (*BcβGLUC*), which degrades both cellulose and hemicellulose (Gilbert, 2010; Blanco-Ulate et al., 2014), and secreted aspartic proteinases (AP) was also highlighted from the qPCR assay (Figure 2.9B). The RNA-Seq data further suggested the involvement of pectin lyases (Bcin03g00280 and Bcin03g07360), enzyme acting preferentially on highly methyl esterified pectin, in the infection process (Supplemental Table S2.9).

The strong upregulation of botcinic acid (*BcBOA*) and botrydial phytotoxins (*BcBOT*) genes, involved in phytotoxins synthesis, pointed towards their participation in the fungal infection program (Figure 2.9C). *BcBOA6* gene encodes a polyketide synthase (PKS), a key enzyme for botcinic acid synthesis (Dalmais et al., 2011), and the *BcBOT1* and *BcBOT2* genes encode P450 monooxygenase and sesquiterpene cyclase, respectively (Siewers et al., 2005).

Taken together, these results indicate the readiness of the fungus to colonize the flowers within 24 hpi. However, the transcript levels of all of the tested infection-related *B. cinerea* genes were much lower at 96 hpi, suggesting that the pathogenesis program initiated at 24 hpi is halted at a later time point. The transcriptional profile of *BcACTA* also suggested that the activity of the fungus decreased towards the later hours of infection. From the post-inoculation inspection of the infected flowers, no visible disease progress was detected until ripening (Figure 2.1). This strengthens the hypothesis that the fungus reduced its biological activity and entered into a quiescent phase.

## 2.5. Discussion

In grapevine, gray mold disease caused by *B. cinerea* occurs mainly on ripe berries close to harvest. The epidemiology of the fungus on grapevine, especially the infection process of the pathogen during flowering, is largely unknown. *B. cinerea* infection of grapevine inflorescence at blooming followed by a "latency period" as a possible source of early bunch rot at véraison was first reported by McClellan and Hewitt (1973). This observation was further confirmed by Keller and colleagues who demonstrated that inoculation at full bloom leads to high disease severity at harvest (Keller et al., 2003). Using the advantage of a GFP-labeled B05.10 strain, we could show for the first time that *B. cinerea* inoculated at flower cap-off stage remained in a quiescent state until berry full coloration (for about two and half months) and then it resumed active growth and invaded the berries when the microclimate was conducive (high humidity). This study provides a detailed description of the infection processes from infection initiation (24 hpi) to initial fungal quiescent (96 hpi) stages by means of transcriptomic and metabolic analyses and microscopic observations, laying the foundation for understanding the mechanism of the plant-fungus interaction during flowering which leads to pathogen quiescence.

Confocal microscopy and transcriptomic studies showed that the fungus, upon contact with the grapevine flowers, attempted to establish infection before becoming quiescent as observed from the germinated appressoria in the flower gynoecium (Figure 2.1B & G) and a prevalent modulation of defense related genes within 24 hpi. The main functional categories of the *in planta* expressed genes of the fungus were those related to pathogenesis, such as CAZymes, oxidative stress, proteases, and transporters (Table 2.3). Interestingly, much of these *in planta* expressed genes were also differentially regulated during successful infection of ripe grapevine

berries (16, 24 and 48 hpi) (Kelloniemi et al., 2015), *Lactuca sativa* (12, 24, and 48 hpi) (De Cremer et al., 2013), and *Solanum lycopersicoides* (24 and 48 hpi) (Smith et al., 2014). This suggests that the *in planta* expressed genes were part of the pathogenesis mechanisms deployed in grapevine flowers that would help to establish infection.

One of the key processes in establishing infection by *B. cinerea* is the depolymerization of cell wall components (van Kan, 2006; Williamson et al., 2007; Zhang et al., 2011). Generally, after breaching the cuticular layer of host tissues, *B. cinerea* often grows into the pectin-rich anticlinal wall of the underlying epidermal cell (van Kan, 2006; Williamson et al., 2007), by the activation of pectinases. In our study, almost all of the assayed CAZymes, including pectinases, were expressed at a higher level at 24 hpi (Figure 2.9). Besides increased levels of CWDEs, the upregulation of genes encoding the biosynthesis of phytotoxic secondary metabolites and secreted proteases, which assist the infection process (Dalmais et al., 2011; Rossi et al., 2011), further confirmed that the fungus put in place several strategies to invade the grapevine flowers. Nonetheless, there was no visible disease symptoms observed in the post-inoculation observations despite the presence of *B. cinerea* was confirmed by the plating out experiment (Figure 2.1H); implying that the pathogen could not grow actively and switched into quiescent phase. The much lower number of *Botrytis* genes expressed *in planta* at 96 hpi as compared to 24 hpi (65% less), as well as the estimated ratio of *B. cinerea* to grapevine RNA (1:500, Supplemental Table S2.2) compared to the much smaller ratio for genomic DNA (about 1:20, Figure 2.1I) in the same tissue, is also a confirmation that the fungus entered the quiescent phase.

Quiescence of a pathogen can happen before conidia germination, at initial hyphal development stage, before or after appressorium formation, after appressorium germination and/or at subcuticular hyphae stage (Prusky, 1996). In unripe tomato, *Colletotrichum gloeosporioides* becomes quiescent as a swollen hyphae after appressorium germination (Alkan et al., 2015), whereas *Alternaria alternata* enters into quiescence after its hypha penetrates the cuticular layer of young apricot, persimmon, and mango fruits (Prusky, 1996). For *B. cinerea*, cell wall penetrated hypha was proposed as a quiescent stage of infection in immature grape berries (Keller et al., 2003). Although the quiescence of ungerminated conidia cannot be completely ruled out in our case, the microscopic observation of conidia germination and appressoria formation (Figure 2.1G and Supplemental Figure S2.3), together with the results of the plating out experiment,

which showed a non-significant effect of washing the inoculated berries (Figure 2.1H), indicate that the pathogen entered into a quiescent state after penetrating the cell wall. The burst of defense related reactions from the plant is also an indirect support for this claim.

In our study the attempted infection by *B. cinerea* instigated a multilayered defense response in the grapevine flower tissues. Following inoculation, more than 70 RLKs were differentially expressed within 24 hpi (Supplemental Table S2.7). Several of these RLKs have been described to be involved in immune response to pathogens. The perception of cell wall fragments, such as oligogalacturonides (OGs) due to CWDE, induces basal resistance to the pathogen (Boller and Felix, 2009). In *A. thaliana*, over-expressing the OG receptor *Wall-associated kinase 1* enhanced resistance to *B. cinerea* (Brutus et al. 2010), but on the other hand increased susceptibility to *Sclerotinia sclerotiorum* and *B. cinerea* was observed in BAK1 mutant *Arabidopsis* (Kemmerling et al., 2007; Zhang et al., 2013). The RLK BAK1 constitutes a negative control element of microbial infection-induced cell death in plants (Kemmerling et al. 2007). These two *RLK*s exhibited increased expression levels in powdery mildew resistant *V. pseudoreticulata* in response to *E. necator* (Weng et al., 2014) and in *B. cinerea* challenged lettuce (De Cremer et al., 2013). We also saw upregulation of genes coding for these membrane receptor proteins at 24 hpi, indicating that the plant recognizes the pathogen and triggers an immunity response: quick and strong induction of PR-proteins and accumulation of stress related secondary metabolites, as well as cell wall lignification are deployed as major defense responses to halt the infection. The oxidative stress caused during the interaction seemed mainly managed by GSTs and ascorbate oxidases (Marrs, 1996) as more than 20 genes coding for them were upregulated at 24 hpi. The pathogen responsive *PR10*, *PR5* (thaumatine-like protein), and chitinases also had a marked upregulation (between 5 and 40-fold upregulation within 24 hpi) compared to the rest of differentially expressed PR-proteins. Chitinases and thaumatin-like proteins are known for their inhibition of fungal growth including *B. cinerea* (Giannakis et al., 1998; Monteiro et al., 2003). From our qPCR result, a very quick induction, as early as 12 hpi, of *VvPR10.1* and *VvPR10.3* was observed. *VvPR10.1* was recently associated to *P. viticola* resistance, under the regulation of *VvWRKY33* (Merz et al., 2015). We demonstrated a coordinated expression of *VvWRKY33* and *VvPR10.1* during *B. cinerea* infection, too. Even though the two pathogens are biologically different and may not necessarily activate similar response from their hosts, *VvPR10.1* and *VvPR10.3*, a PR belonging to the same group of *VvPR10.1* (Lebel et al., 2010), had the highest

upregulation among the expressed PRs, making them strong candidates for the resistance of grapevine flower to *B. cinerea*. In *Arabidopsis WRKY33*, the functional homologue of *VvWRKY33* (Merz et al., 2015), plays a key role in the plant defense process, regulating redox homeostasis, SA signaling, ET-JA-mediated cross-communication, and phytoalexin biosynthesis conferring resistance to *B. cinerea* (Birkenbihl et al., 2012).

Upon *B. cinerea* infection, grapevine berries activate stilbenoid biosynthesis (Langcake, 1981; Jeandet et al., 1995; Keller et al., 2003; Agudelo-Romero et al., 2015; Kelloniemi et al., 2015). Our results were in line with previous evidence: many genes involved in stilbenoid and flavonoid biosyntheses were upregulated, STS and laccase coding genes were the ones with the highest upregulation. From the RNA-Seq results, it seemed that the genes coding for STS and laccase proteins were switched on following the infection, since most of them were below the detection limit in the mock-inoculated flowers. The activation of the polyphenol biosynthetic pathway was further investigated by a more fine-grained expression profile of the transcription factor regulating stilbene biosynthesis (*VvMYB14*, Höll et al., 2013) together with *VvSTS29* and *VvSTS41*, and by measuring the concentration of several classes of polyphenols (phenylpropanoids, stilbenoids, and flavonoids). The phytoalexin resveratrol, which inhibits *B. cinerea* growth (Schouten et al., 2002b; Favaron et al., 2009) and its monomeric and oligomeric derivatives, some with documented antifungal activity (Hammerbacher et al., 2011), were all upregulated starting from 12 hpi. The concentrations of ε-viniferin and α-viniferin, dimer and trimer resveratrol respectively, were higher in *Botrytis*-infected flowers than control throughout the measured period. These compounds represent the predominant stress metabolites in *Vitaceae* - *B. cinerea* interactions reported previously (Langcake, 1981). We also observed a marked oligomerization of resveratrol to ampelopsin D and quadrangularin, E-cis-miyabenol, Z-miyabenol C, and isohopeaphenol upon infection, which agrees with the hypothesis that oligomerization of resveratrol leads to more toxic compounds (Pezet et al., 2003a). On the other hand, overexpressing the glycosylated form of resveratrol, trans-piceid, in *Arabidopsis* that overexpresses a resveratrol gene *PcRS*, increased its resistance to *Colletotrichum higginsianum* (Liu et al., 2011). In line with this, over-expression of an *STS* gene in transgenic grapevine plants led to resistance to *B. cinerea* colonization (Coutos-Thévenot et al., 2001; Dabauza et al., 2014) and a strong correlation was also observed between the concentration of stilbenic phytoalexins

and resistance to *P. viticola* both in a *V. riparia* and *Merzling* × *Teroldego* cross population (Langcake, 1981; Malacarne et al., 2011).

The potent stilbene oxidase inhibitors caftaric and *trans*-coutaric acids (both phenylpropanoids), catechin, and quercetin-3-O-glucuronide (both flavonoids) (Goetz et al., 1999) were also detected at higher concentrations in the *Botrytis*-infected flowers than in the control, contributing to the defense reaction. These compounds possibly interfered with the fungal laccase activity by inactivating its oxidizing and insolubilizing effects on stilbenic phytoalexins and PR proteins (Goetz et al., 1999; Favaron et al., 2009). In addition, different classes of flavonoids including proanthocyanidins (procyanidins B1, B2, B3 and B4) were also detected at a higher concentration in the infected flowers; these compounds can act as inhibitors of enzymes such as polygalacturonases. Higher concentration of proanthocyanidins in the epidermal layer of immature strawberry, at the periphery of *B. cinerea* penetration, was reported to restrict further growth of *B. cinerea* and keep the pathogen under quiescence (Jersch et al., 1989). Taken together, our results indicated a dramatic and rapid accumulation of the polyphenolic metabolites, in particular stilbenoids, at the site of infection suggesting this is a mechanism of defense to induce *B. cinerea* quiescence in grapevine flower.

Reinforcing cell walls is another strategy of plant resistance against pathogens. From our microscopic observations, cell wall apposition occurred at the appresorium penetration site (Figure 2.1F and Figure 2.8A). Gene expression and metabolite analysis also indicated that grapevine flowers, upon *B. cinerea* infection, activated the monolignol biosynthesis pathway within 24 hpi. A significant accumulation of the metabolites at the beginning of the pathway (L-phenylalanine and cinnamate) as well as at the end of the pathway (sinapaldehyde and coniferyl alcohol) was indeed observed. Instead, whereas, most of the intermediates were very low (such as p-coumarate and caffeate) or not detectable, possibly due to their rapid conversion into products for the next steps in the pathway. A similar phenomenon occurs in wheat, where upregulation of monolignol genes within 24 hpi conferred resistance to *Blumeria graminis* f. sp. *Tritici*; and silencing a few key genes in the pathway (PAL, COMT, CCoAMT and CAD) compromised penetration resistance of the plant to the pathogen (Bhuiyan et al., 2009). The upregulation of *GLP3* and *EXT*, both at 24 and 96 hpi, and accumulation of $H_2O_2$ around the penetration site, as shown using the HyPer signal (Figure 2.5), suggests CWA was triggered as an early response to

the infection. $H_2O_2$-mediated oxidative cross-linking and lignin synthesis to reinforce CWA and halt *B. cinerea* infection was recently shown in grapevine by Kelloniemi et al. (2015). In their study, the upregulation of lignin forming enzymes (GLP3 and EXT) together with the accumulation of $H_2O_2$, at the site of *Botrytis* penetration, were part of the defense mechanisms used by the véraison berry to arrest the pathogen. However, no such queues of responses were seen in the mature berry where the pathogen readily managed to colonize the berry tissue (Kelloniemi et al., 2015). In green tomatoes, usually resistant to *B. cinerea*, the accumulation of $H_2O_2$ and lignin occurrs at the site of inoculation (Cantu et al., 2009), and in a tomato *sitiens* mutant, primed $H_2O_2$ accumulation and cell wall reinforcement were among the resistant factors against *B. cinerea* (Asselbergh et al., 2007). These all show that the CWA-mediated resistance was also active in the *B. cinerea* infected flowers.

## 2.6. Conclusions

Flowering is a critical phenological stage in the epidemiology of *B. cinerea* in the vineyard leading to quiescent infection. Our work provides the first transcriptomic and targeted metabolites analyses of the interaction between the two organisms at this critical phenological stage. Our results showed that upon contact with the grapevine flower, within 24 hpi, *B. cinerea* induced genes encoding for CWDE, phytotoxic secondary metabolites, and proteases, indicating a readiness to establish a successful infection. There was no visible disease symptom in the post inoculation time however, despite the confirmed presence of the pathogen and an egression at the later ripening stage. Flowers reacted readily to the infection as their defense mechanisms were very in-line upon recognizing the intruder. There was a marked accumulation of antimicrobial proteins (mainly PR-proteins), monolignol precursors, stilbenoids, and reactive oxygen species accompanied by cell wall reinforcement. The conjugated actions of these induced defense responses seem to be responsible for forcing *B. cinerea* into quiescence until more favorable conditions occur in the berry.

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## 2.8. Supplemental materials

### 2.8.1. Supplemental tables

Supplemental Table S2.1. List of qPCR primers. Gene and/or accession identification, gene name, primer name, primer sequence, and source are listed.

| | Gene/Accession ID | Gene name | Primer name | Primer sequence | Reference |
|---|---|---|---|---|---|
| *Botrytis cinerea* | AM233400.1 | *Bc3* | Bc3-F | TGTAATTTCAATGTGCAGAATCC | Suarez et al. 2005 |
| | | | Bc3-R | TTGAAATGCGATTAATTGTTGC | |
| | Bcin16g02020 | *BcACTA* | BcactA-F | CGTCACTACCTTCAACTCCATC | Li et al. 2014 |
| | | | BcactA-R | CGGAGATACCTGGGTACATAGT | |
| | Bcin07g03040 | *BcAOX* | Bcaox-F | GTTCTCAAAGGGGTCTGCTG | This study |
| | | | Bcaox-R | GTTCCTACCGAGTGCCATGT | |
| | Bcin12g02040 | *BcAP8* | Bcap8-F | CATCGTCGATACTGGATCCTC | ten Have et al. 2010 |
| | | | Bcap8-R | TGAACCACTTCCGTAGGAGAC | |
| | Bcin12g00180 | *BcAP9* | Bcap9-F | AGCAAGGTTCAAGGTGCTGT | This study |
| | | | Bcap9-R | CACGGACGGTAGCCATGTAG | |
| | Bcin01g00060 | *BcBOA6* | BcPks6q-F | CAGCAATCGTTGTCCTGAAATC | Schumacher et al. 2015 |
| | | | BcPks6q-R | GTTTATCGCGTTCTTCACCTGTTA | |
| | Bcin12g06380 | *BcBOT1* | Bcbot1-F | GGCTCCCGTGCTTGCA | Pinedo et al. 2008 |
| | | | Bcbot1-R | GCGAGGTGAAGAAGTTAGAGAAGGT | |
| | Bcin12g06390 | *BcBOT2* | Bcbot2 F | CAGGTTATCCCTTTGCATGAGTAGT | Pinedo et al. 2008 |
| | | | Bcbot2 R | TTACACTGGTGAATGATGTTTTGTCTT | |
| | Bcin15g03080 | *BcCUTA* | BccutA-F | TGCTGGCAGTCAGACTATGG | This study |
| | | | BccutA-R | TTCGGCTGGTAAAAGTTTGG | |
| | Bcin01g09430 | *BcCUT-like1* | Bccut-like1-F | TCACCAACTACGCTTCCACC | This study |
| | | | Bccut-like1-R | GCAATCTTGGCCGTTACAGC | |
| | Bcin03g01500 | *BcGAR2* | Bcgar2-F | CCCAGCTATCCGTGAACATC | Zhang et al. 2011 |
| | | | Bcgar2-R | CACCTGGGGAAAGCGCATC | |
| | Bcin03g01490 | *BcGLA1* | Bcgla1-F | CAAGGTTTGGGAATTGTACAGAG | Zhang et al. 2011 |
| | | | Bcgla1-R | GTATCCTCCATATCCATAGTAGC | |
| | Bcin13g05710 | *BcGOX1* | Bcgox1-F | AAGGGTTTTGAATGCAGGTG | This study |
| | | | Bcgox1-R | AGCTTCCCTTCGTCTGTCAA | |
| | Bcin03g01480 | *BcGPX3* | Bcgpx3-F | GGTGACAATGCTGCTCCTCT | This study |
| | | | Bcgpx3-R | TCTGGCTTGGTTGTGGATGC | |
| | Bcin10g00740 | *BcGST1* | Bcgst1-F | GTTGAGAAGGGCCGTCATGT | Kolloniemi et al. 2015 |
| | | | Bcgst1-R | CCTCACGTTCAGGGTCTTTCTT | |
| | Bcin14g02510 | *BcLCC2* | Bclcc2-F | TGCCCTCACTGCATTATTTG | This study |
| | | | Bclcc2-R | CTGGAAGTAGCCGAGTTTGC | |
| | Bcin01g09450 | *BcLGD1* | Bclgd1-F | TGGTCATGGCATGACTTTCAC | Zhang et al. 2011 |
| | | | Bclgd1-R | GTTGCGAATCGGAAACGAGATA | |
| | Bcin12g01020 | *BcOAH* | Bcoah-F | CGACGAGTGCATCAAGAGGT | This study |
| | | | Bcoah-R | AACAGTCTTTGCGGCCATCT | |
| | Bcin03g05820 | *BcPEL-like1* | Bcprl-like1-F | ACCACCACTGTCTCCACCTA | This study |
| | | | Bcpel-like1-R | CCTTGACTTGGGAAGCAGCT | |
| | Bcin14g00850 | *BcPG1* | Bcpg1-F | CTGCCAACGGTGTCCGTATC | Zhang and vanKan 2013 |
| | | | Bcpg1-R | GAACGACAACACCGTAGGATG | |
| | Bcin14g00610 | *BcPG2* | Bcpg2-F | CGAGTTAAGACCGTCAGCGATA | This study |
| | | | Bcpg2-R | CGATGAGAGTGATGTCCTGGAA | |
| | Bcin03g01680 | *BcPG4* | Bcpg4-F | CTTATTGAGTACGCCACTGTC | Zhang and vanKan 2013 |
| | | | Bcpg4-R | AGTGTCGACGGTGTTGTTGC | |

Supplemental Table S2.1. Continued

| | Gene/Accession ID | Gene name | Primer name | Primer sequence | Reference |
|---|---|---|---|---|---|
| *Botrytis cinerea* | Bcin02g05860 | *BcPG6* | Bcpg6-F | ATTTGATGTCAGCTCGTCCAG | Zhang and vanKan 2013 |
| | | | Bcpg6-R | ACCTGAGCAATATAACCCGTC | |
| | Bcin13g05720 | *BcPRD1* | Bcprd1-F | ACCCAGCAATTGAGTTCACC | This study |
| | | | Bcprd1-R | CCTTGGGTGTCACCTCATCT | |
| | Bcin01g09620 | *BcRPL5* | Bcrpl5-F | GATGAGACCGTCAAATGGTTC | Zhang and vanKan, 2013 |
| | | | Bcrpl5-R | CAGAAGCCCACGTTACGACA | |
| | Bcin03g03390 | *BcSOD1* | BcSod1-F | CCATCAATTCGGTGACAACACT | Kelloniemi et al. 2015 |
| | | | BcSod1-R | TGGCCGTGTGGGTTGAA | |
| | Bcin01g08040 | *BcTUBA* | BctubA-F | TTTGGAGCCAGGTACCATGG | Mehli et al. 2005 |
| | | | BctubA-R | GTCGGGACGGAAGAGTTGAC | |
| | Bcin03g00480 | *BcXYN11A* | Bcxyn-F | CCTGGAAGAAGTTGGGATTG | This study |
| | | | Bcxyn-R | AACAGTGATGGAAGCGGAAC | |
| | Bcin10g05590 | *BcβGLUC* | Bcβgluc-F | TGCAGCTACCTTTGATCGTG | This study |
| | | | Bcβgluc-R | TCCTTCCCAGTTACGTCCAC | |
| *Vitis vinifera* | VIT_11s0052g01110 | *Vv4CL* | Vv4CL-F | TTCCCGACATCAACATCCCG | This study |
| | | | Vv4CL-R | TTACGTGCGGTGAGATGGAC | |
| | VIT_04s0044g00580 | *VvACT* | VvACT-F | ATGTGCCTGCCATGTATGTTGCC | Bèzier et al. 2002 |
| | | | VvACT-R | AGCTGCTCTTTGCAGTTTCCAGC | |
| | VIT_11s0065g00350 | *VvC4H* | VvC4H-F | ATTGACGTGTCCGAAAAAGG | This study |
| | | | VvC4H-R | CTATGCGGTGATTGGAGTGA | |
| | VIT_03s0180g00250 | *VvCAD* | VvCAD-F | GTGGAGGTGGGATCAGATGT | This study |
| | | | VvCAD-R | TCCATCTCTGATTTGCATGG | |
| | VIT_03s0063g00140 | *VvCCoAOMT* | VvCCoAOMT-F | TCGATTTGGTGAAGGTGGGG | This study |
| | | | VvCCoAOMT-R | AGAGCCTTGTTCAGCTCCAA | |
| | VIT_14s0066g01150 | *VvCCR* | VvCCR-F | AGCAGAAACAGGGATGCCAT | This study |
| | | | VvCCR-R | AGAGAGCCTCCCATCTGACA | |
| | VIT_05s0094g00360 | *VvCHIT4c* | VvCHIT4c-F | TCGAATGCGATGGTGGAAA | Ramírez-Suero et al. 2014 |
| | | | VvCHIT4c-R | TCCCCTGTCGAAACACCAAG | |
| | VIT_16s0098g00850 | *VvCOMT* | VvCOMT-F | GCCTTCTTGCCACCTATGCT | This study |
| | | | VvCOMT-R | TCATGAGGAGAAGAGGGGCT | |
| | VIT_07s0031g01380 | *VvF5H* | VvF5H-F | CTTTGTGCCCGCCATTGTTG | This study |
| | | | VvF5H-R | TTGAACACTCATGGGGTGGC | |
| | VIT_14s0060g00120 | *VvGLP2* | VvGLP2-F | CGAGTTGGATGTGGGGTTCA | Godfrey et al. 2007 |
| | | | VvGLP2-R | GACTTCGCCGTTGTTCTTCT | |
| | VIT_19s0093g00320 | *VvGST1* | VvGST1-F | CCAAAGAGCAAAAGCCAAGT | Conn et al. 2008 |
| | | | VvGST1-R | TGTCCAGAAAACCCAAAGTC | |
| | VIT_01s0146g00480 | *VvJAZ10* | VvJAZ10-F | TCCGAAGAATAATCCGCCGT | This study |
| | | | VvJAZ10-R | CAGGACTGTAAACCGGCAAC | |
| | VIT_07s0005g03340 | *VvMYB14* | VvMYB14-F | TCTGAGGCCGGATATCAAAC | Höll et al. 2013 |
| | | | VvMYB14-R | GGGACGCATCAAGAGAGTGT | |
| | VIT_01s0010g02020 | *VvPER* | VvPER-F | AGGGCAAGCAAGATGTGTGA | This study |
| | | | VvPER-R | TCCAGGGGTGCAAGATTGTC | |
| | VIT_08s0007g08330 | *VvPG1* | VvPG1-F | CAAGCGAGCCCACCTTATGA | This study |
| | | | VvPG1-R | CTCACGGCTTTGAATGGTGC | |
| | VIT_08s0040g00550 | *VvPLR* | VvPLR-F | AAAGGCCGGATATGGGTGTG | This study |
| | | | VvPLR-R | TCTACCAACTTCACGGCGTC | |
| | VIT_03s0088g00750 | *VvPR1* | Vv_PR1i-F | CTCCCCTTCATTGACAGGCA | This study |
| | | | Vv_PR1i-R | ATAGTGCCTGCATTCCCCAC | |
| | VIT_03s0088g00710 | *VvPR1* | Vv_PR1ii-F | GCGTGGGTGGGGAATGCCGA | Trdá et al. 2014 |
| | | | Vv_PR1ii-R | GATGTTGTCCCTGATAGTTGCC | |

Supplemental Table S2.1. Continued

| | Gene/Accession ID | Gene name | Primer name | Primer sequence | Reference |
|---|---|---|---|---|---|
| *Vitis vinifera* | VIT_05s0077g01530 | *VvPR10.1* | VvPR10.1-F | GCACATCCCGATGCCTATTAAG | Merz et al. 2015 |
| | | | VvPR10.1-R | ACTTACTGAGACTGATAGATGCAATGAATA | |
| | VIT_05s0077g01560 | *VvPR10.3* | VvPR10.3-F | GAAATCCTACAAGGACAGGGAGGT | Lebel et al. 2010 |
| | | | VvPR10.3-R | CGGCCTTGGTGTGGTACTTTT | |
| | AF274281 | *VvRS I* | VvRES I- F | CGAGGAATTTAGAAACGCTCAAC | Valsesia et al. 2005 |
| | | | VvRES I- R | GCTGTGCCAATGGCTAGGA | |
| | VIT_08s0058g00790 | *VvSIRD* | VvSIRD-F | TGAAGACACACTCGGAGCTG | This study |
| | | | VvSIRD-R | AGGATATCGAGGCGTCCGTA | |
| | VIT_16s0100g00950; VIT_16s0100g00990; VIT_16s0100g01010 | *VvST29 (VvST25+VvST27+VvST29)* | VvST29-F | GGTTTTGGACCAGGCTTGACT | Höll et al. 2013 |
| | | | VvST29-R | GAGATAAATACCTTACTCCTATTCAAC | |
| | VIT_16s0100g01130; VIT_16s0100g01160 | *VvST41 (VvST41 + VvST45)* | VvST41-F | GAGTACTATTTGGTTTTGGACCT | Höll et al. 2013 |
| | | | VvST41-R | AACTCCTATTTGATACAAAACAACGT | |
| | VIT_06s0004g00480 | *VvTUB* | VvTUB-F | TGTTGGTGAAGGCATGGAGG | Giacomelli et al. 2012 |
| | | | VvTUB-R | AGATGACACGCCTGCTGAACT | |
| | VIT_08s0058g00690 | *VvWRKY33* | VvWRKY33-F | ATTCAAGCACTAGTATGAACAGAGCAG | Merz et al. 2015 |
| | | | VvWRKY33-R | CCTTGTTGCCTTGGCATGA | |

Supplemental Table S2.2. Summary of reads mapping of the 15 RNA-Seq libraries. Ctrl, mock inoculated; Trt, *B. cinerea* inoculated; Bc, *Botrytis cinerea*; 1-3 indicate the three biological replicates; 24 and 96 indicate hours post inoculation.

| Library | Total quality-trimmed reads | Reads mapped to *V. vinifera* reference | Reads uniquely mapped to *V. vinifera* reference | Reads mapped to *B. cinerea* reference | Reads uniquely mapped to *B. cinerea* reference |
|---|---|---|---|---|---|
| Ctrl 1-24 | 33,582,861 | 26,185,827 (77.97 %) | 24,938,558 (74.26 %) | 60,629 (0.18 %) | 9,736 (0.03 %) |
| Ctrl 2-24 | 30,634,590 | 24,156,916 (78.86 %) | 23,009,237 (75.11 %) | 63,570 (0.21 %) | 10,476 (0.03 %) |
| Ctrl 3-24 | 28,509,351 | 21,919,491 (76.89 %) | 20,541,510 (72.05 %) | 301,999 (1.06 %) | 26,834 (0.09 %) |
| Ctrl 1-96 | 22,410,851 | 16,848,719 (75.18 %) | 15,496,486 (69.15 %) | 472,766 (2.11 %) | 7,195 (0.03 %) |
| Ctrl 2-96 | 37,394,962 | 24,336,710 (65.08 %) | 23,155,616 (61.92 %) | 60,179 (0.16 %) | 7,598 (0.02 %) |
| Ctrl 3-96 | 27,843,357 | 21,949,631 (78.83 %) | 20,838,437 (74.84 %) | 92,550 (0.33 %) | 11,458 (0.04 %) |
| Trt 1-24 | 27,812,146 | 19,069,689 (68.57 %) | 16,282,036 (58.54 %) | 1,281,100 (4.6 %) | 35,213 (0.12 %) |
| Trt 2-24 | 31,644,086 | 25,622,237 (80.97 %) | 24,469,696 (77.33 %) | 134,816 (0.43 %) | 72,414 (0.23 %) |
| Trt 3-24 | 30,211,586 | 24,631,219 (81.53 %) | 23,391,950 (77.43 %) | 164,851 (0.55 %) | 77,040 (0.26 %) |
| Trt 1-96 | 25,919,449 | 21,240,182 (81.95 %) | 20,270,161 (78.20 %) | 77,351 (0.34 %) | 16,453 (0.06 %) |
| Trt 2-96 | 26,077,701 | 20,388,725 (78.18 %) | 19,359,716 (74.24 %) | 116,774 (0.45 %) | 54,139 (0.21 %) |
| Trt 3-96 | 23,503,720 | 18,957,227 (80.66 %) | 18,077,115 (76.91 %) | 79,818 (0.34 %) | 28,891 (0.12 %) |
| Bc 1 | 22,223,388 | 76,740 (0.35 %) | 21,423 (0.01 %) | 20,072,229 (90.32 %) | 14,108,503 (63.48 %) |
| Bc 2 | 21,289,297 | 47,738 (0.22 %) | 28,559 (0.13 %) | 19,256,732 (90.45 %) | 16,603,159 (77.99 %) |
| Bc 3 | 22,254,222 | 50,719 (0.23 %) | 17,798 (0.08 %) | 20,086,452 (90.25 %) | 16,522,732 (74.24 %) |

Supplemental Table S2.3. Differentially expressed genes of *V. vinifera* flowers upon *B. cinerea* infection (Provided as excel file)

Supplemental Table S2.4. List of genes used to validate the RNA-Seq expression values by q-PCR assay. Gene identification, gene description, and fold change from RNA-seq and qPCR are provided.

| Gene ID | Description | RNA-seq (Log2 fold change) | | qPCR (Log2 fold change) | |
|---|---|---|---|---|---|
| | | 24 hpi | 96 hpi | 24 hpi | 96 hpi |
| VIT_04s0044g00580 | Actin 7 (ACT7) / actin 2 | -0.25 | 0.02 | -0.05 | -0.04 |
| VIT_06s0004g00480 | Tubulin alpha | 0.05 | 0.10 | 0.05 | 0.04 |
| VIT_08s0058g00690 | WRKY DNA-binding protein 33 | 1.65 | -0.04 | 1.14 | -0.25 |
| VIT_14s0060g00120 | Germin-like protein 2 | 0.27 | -0.04 | 0.40 | -0.13 |
| VIT_07s0005g03340 | Myb domain protein 14 | 1.87 | -0.05 | 1.46 | -0.32 |
| VIT_03s0088g00750 | Pathogenesis related protein 1 precursor | 1.45 | 0.21 | 1.07 | -0.15 |
| VIT_03s0088g00710 | Pathogenesis-related protein 1 precursor | 0.75 | 0.34 | 0.80 | 0.39 |
| VIT_05s0077g01530 | Pathogenesis protein 10 [Vitis vinifera] | 4.94 | 1.56 | 3.89 | 2.13 |
| VIT_19s0093g00320 | Glutathione S-transferase 25 GSTU25 | 2.11 | 0.56 | 1.57 | 1.30 |
| VIT_05s0094g00360 | Chitinase class IV | 2.35 | 0.23 | 1.90 | 0.49 |
| VIT_01s0146g00480 | TIFY 9 (Jasmonate ZIM domain-containing protein 10) | 1.06 | -0.82 | 1.07 | -0.43 |
| VIT_08s0007g08330 | Polygalacturonase PG1 | -4.80 | -2.40 | -3.18 | -1.07 |
| VIT_01s0010g02020 | Peroxidase | 7.12 | 2.09 | 5.46 | 2.77 |
| VIT_07s0031g01380 | Ferulate 5-hydroxylase | 2.04 | 0.96 | 2.48 | 1.47 |
| VIT_11s0052g01110 | 4-coumarate-CoA ligase 1 | 1.96 | 0.06 | 2.20 | -0.27 |
| VIT_03s0063g00140 | Caffeoyl-CoA O-methyltransferase | 1.33 | 0.25 | 1.43 | 0.19 |
| VIT_16s0098g00850 | Caffeic acid O-methyltransferase | 0.68 | -0.06 | 0.91 | 0.34 |
| VIT_08s0040g00550 | Pinoresinol-lariciresinol reductase | 1.37 | -0.63 | 1.28 | 0.08 |
| VIT_14s0066g01150 | Cinnamoyl-CoA reductase | 1.15 | 0.05 | 1.35 | 0.74 |
| VIT_08s0058g00790 | Secoisolariciresinol dehydrogenase | 1.51 | 0.31 | 1.77 | 1.08 |

Supplemental Table S2.5. 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. BP, biological process; MF, molecular function; CC, cellular component.

| Hours post inoculation | GO term | Ontology | Description | No. of genes in reference set with any GO term assigned | No. of genes in test set with any GO term assigned | Number in input list | Number in reference list | p-value | FDR | Enrichment |
|---|---|---|---|---|---|---|---|---|---|---|
| 24 | GO:0019748 | BP | secondary metabolic process | 23,531 | 1,092 | 87 | 374 | 8.00E-35 | 2.60E-32 | 5.0 |
| 24 | GO:0009607 | BP | response to biotic stimulus | 23,531 | 1,092 | 35 | 233 | 1.50E-09 | 6.90E-08 | 3.2 |
| 24 | GO:0009875 | BP | pollen-pistil interaction | 23,531 | 1,092 | 24 | 185 | 6.70E-06 | 1.70E-04 | 2.8 |
| 24 | GO:0006464 | BP | protein modification process | 23,531 | 1,092 | 147 | 2,097 | 2.60E-06 | 8.70E-05 | 1.5 |
| 24 | GO:0007049 | BP | cell cycle | 23,531 | 1,092 | 25 | 193 | 4.50E-06 | 1.30E-04 | 2.8 |
| 24 | GO:0009056 | BP | catabolic process | 23,531 | 1,092 | 81 | 1,084 | 4.10E-05 | 8.80E-04 | 1.6 |
| 24 | GO:0003774 | MF | motor activity | 23,531 | 1,092 | 25 | 118 | 2.10E-10 | 4.40E-09 | 4.6 |
| 24 | GO:0030246 | MF | carbohydrate binding | 23,531 | 1,092 | 40 | 403 | 8.60E-06 | 1.50E-04 | 2.1 |
| 24 | GO:0016301 | MF | kinase activity | 23,531 | 1,092 | 132 | 2,132 | 1.10E-03 | 9.50E-03 | 1.3 |
| 24 | GO:0004871 | MF | signal transducer activity | 23,531 | 1,092 | 98 | 1,475 | 4.90E-04 | 5.80E-03 | 1.4 |
| 24 | GO:0016740 | MF | transferase activity | 23,531 | 1,092 | 263 | 4,541 | 6.40E-04 | 6.80E-03 | 1.2 |
| 24 | GO:0005576 | CC | extracellular region | 23,531 | 1,092 | 73 | 887 | 4.10E-06 | 5.60E-04 | 1.8 |
| 96 | GO:0005576 | CC | extracellular region | 23,531 | 244 | 31 | 887 | 2.60E-08 | 1.80E-06 | 3.4 |
| 96 | GO:0005618 | CC | cell wall | 23,531 | 244 | 15 | 319 | 2.40E-06 | 8.10E-05 | 4.5 |

Supplemental Table S2.6. MapMan BINs enriched in the differentially expressed grapevine genes upon B. cinerea infection. Over- and under-represented functions are presented. NA, not available.

| Hours post inoculation | MapMan Bin code | MapMan Bin Name | Representation | FDR |
|---|---|---|---|---|
| 24 | 16 | secondary metabolism | overrepresented | <1E-20 |
| 24 | 16.1 | secondary metabolism.isoprenoids | overrepresented | 6.11E-07 |
| 24 | 16.1.5 | secondary metabolism.isoprenoids.terpenoids | overrepresented | 1.26 E-2 |
| 24 | 16.2 | secondary metabolism.phenylpropanoids | overrepresented | 1.02E-04 |
| 24 | 16.2.1 | secondary metabolism.phenylpropanoids.lignin biosynthesis | overrepresented | 4.89E-05 |
| 24 | 16.2.1.1 | secondary metabolism.phenylpropanoids.lignin biosynthesis.PAL | overrepresented | 2.17E-05 |
| 24 | 16.8 | secondary metabolism.flavonoids | overrepresented | 1.55E-14 |
| 24 | 16.8.2 | secondary metabolism.flavonoids.chalcones | overrepresented | <1E-20 |
| 24 | 16.8.2.3 | Secondary metabolism.flavonoids.chalcones.stilbene synthase | overrepresented | <1E-20 |
| 24 | 20 | stress | overrepresented | 1.75 E-2 |
| 24 | 20.1 | stress.biotic | overrepresented | 9.40 E-2 |
| 24 | 20.1.7 | stress.biotic.PR-proteins | overrepresented | 2.07E-04 |
| 24 | 26 | misc | overrepresented | 9.75E-05 |
| 24 | 26.8 | nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases | overrepresented | 2.11 E-3 |
| 24 | 26.9 | misc.glutathione S transferases | overrepresented | 6.25 E-2 |
| 24 | 27.3.32 | RNA.regulation of transcription.WRKY domain transcription factor family | overrepresented | 9.07 E-2 |
| 24 | 31 | cell | underrepresented | 3.08E-07 |
| 24 | 31.1 | cell.organisation | underrepresented | 2.28E-06 |
| 24 | 30 | signalling | overrepresented | 3.67 E-2 |
| 24 | 30.2 | signalling.receptor kinases | overrepresented | 7.54 E-2 |
| 24 | 30.2.99 | signalling.receptor kinases.misc | overrepresented | 3.24 E-3 |
| 96 | NA | NA | NA | NA |

Supplemental Table S2.7. List of differentially expressed *V. vinifera* genes belonging to the biotic stress functional categories. (Provided as excel file)

Supplemental Table S2.8. Concentration of polyphenolic secondary metabolites in mock (Ctrl) and B. cinerea (Trt) inoculated grapevine flowers. The amount is given in µg/g fresh weight. LOD, limit of detection; hpi, hour post inoculation; SE, standard error.

| Secondary metabolites | LOD(µg/g) | 12 hpi | | | | 24 hpi | | | | 48 hpi | | | | 72 hpi | | | | 96 hpi | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ctrl | | Trt | | Ctrl | | Trt | | Ctrl | | Trt | | Ctrl | | Trt | | Ctrl | | Trt | |
| | | mean | SE | Trt | SE | mean | SE | Trt | SE | mean | SE | Trt | SE | mean | SE | Trt | SE | mean | SE | Trt | SE |
| *p*-hydroxybenzoic acid | 0.005 | 0.6 | 0.06 | 1.0 | 0.15 | 0.5 | 0.09 | 1.2 | 0.26 | 1.4 | 0.06 | 1.6 | 0.24 | 0.8 | 0.18 | 1.7 | 0.37 | 0.7 | 0.02 | 1.8 | 0.42 |
| vanillic acid | 0.0025 | 0.1 | 0.01 | 0.1 | 0.05 | 0.0 | 0.01 | 0.0 | 0.00 | 0.1 | 0.01 | 0.1 | 0.01 | 0.1 | 0.00 | 0.1 | 0.01 | 0.1 | 0.01 | 0.1 | 0.01 |
| gallic acid | 0.025 | 5.0 | 0.87 | 9.1 | 4.25 | 4.8 | 1.28 | 12.1 | 3.25 | 8.7 | 0.46 | 13.4 | 1.49 | 3.6 | 4.82 | 18.0 | 1.36 | 5.9 | 0.78 | 19.5 | 5.57 |
| 2,6-diOH-benzoic acid | 0.0025 | 0.1 | 0.03 | 0.1 | 0.02 | 0.1 | 0.06 | 0.1 | 0.06 | 0.1 | 0.03 | 0.1 | 0.04 | 0.1 | 0.01 | 0.1 | 0.02 | 0.0 | 0.00 | 0.2 | 0.09 |
| fraxin | 0.0025 | 0.1 | 0.01 | 0.2 | 0.03 | 0.1 | 0.01 | 0.2 | 0.08 | 0.2 | 0.02 | 0.2 | 0.02 | 0.1 | 0.09 | 0.2 | 0.05 | 0.1 | 0.03 | 0.1 | 0.01 |
| caftaric acid | 0.0125 | 444.7 | 98.32 | 630.4 | 204.68 | 268.9 | 35.17 | 646.4 | 182.70 | 584.5 | 35.48 | 929.0 | 56.76 | 450.3 | 207.44 | 949.7 | 203.86 | 579.7 | 47.01 | 1227.7 | 408.41 |
| fertaric acid | 0.0025 | 59.0 | 4.51 | 85.6 | 19.15 | 54.0 | 6.65 | 116.4 | 33.16 | 150.5 | 2.57 | 176.4 | 17.75 | 107.7 | 3.28 | 135.1 | 11.17 | 42.0 | 2.27 | 116.3 | 16.46 |
| *trans*-coutaric acid | 0.025 | 118.6 | 10.38 | 177.3 | 36.59 | 73.3 | 14.57 | 184.8 | 51.64 | 175.2 | 13.95 | 267.4 | 25.73 | 135.4 | 53.47 | 257.5 | 49.83 | 115.6 | 0.19 | 297.5 | 59.57 |
| phlorizin | 0.0025 | 2.3 | 0.26 | 3.5 | 1.08 | 1.6 | 0.26 | 4.3 | 1.21 | 3.8 | 0.13 | 6.1 | 1.07 | 2.3 | 2.59 | 7.6 | 2.19 | 2.4 | 0.06 | 6.4 | 1.88 |
| luteolin | 0.0025 | 0.1 | 0.01 | 0.2 | 0.05 | 0.0 | 0.01 | 0.1 | 0.02 | 0.1 | 0.01 | 0.1 | 0.03 | 0.1 | 0.01 | 0.1 | 0.02 | 0.0 | 0.02 | 0.1 | 0.04 |
| luteolin-7-O-Glc | 0.0025 | 1.3 | 0.14 | 1.7 | 0.55 | 0.6 | 0.17 | 2.0 | 0.58 | 1.6 | 0.13 | 2.5 | 0.21 | 1.0 | 0.60 | 2.7 | 0.67 | 1.2 | 0.15 | 3.2 | 1.01 |
| naringenin | 0.0025 | 0.3 | 0.04 | 0.6 | 0.11 | 0.1 | 0.02 | 0.4 | 0.04 | 0.4 | 0.15 | 0.4 | 0.10 | 0.4 | 0.05 | 0.4 | 0.02 | 0.5 | 0.12 | 0.8 | 0.42 |
| catechin | 0.025 | 39.9 | 5.36 | 61.3 | 15.69 | 61.1 | 7.02 | 136.4 | 27.12 | 141.3 | 13.07 | 136.2 | 25.71 | 97.7 | 2.03 | 207.1 | 44.66 | 86.0 | 5.65 | 158.9 | 26.18 |
| epicatechin | 0.025 | 3.9 | 0.57 | 5.6 | 1.34 | 5.1 | 0.86 | 10.3 | 2.14 | 13.5 | 0.46 | 13.1 | 2.00 | 9.8 | 0.40 | 17.4 | 3.13 | 8.0 | 1.74 | 18.7 | 0.43 |
| epigallocatechin | 1.25 | 2.6 | 0.10 | 4.1 | 0.97 | 2.4 | 0.22 | 4.7 | 1.14 | 4.9 | 0.15 | 5.5 | 0.53 | 3.5 | 0.93 | 7.2 | 1.54 | 3.3 | 0.08 | 6.0 | 1.11 |
| gallocatechin | 0.25 | 2.2 | 0.07 | 2.4 | 0.62 | 2.2 | 0.57 | 3.9 | 0.19 | 2.8 | 0.17 | 4.0 | 0.67 | 2.9 | 0.53 | 8.4 | 2.25 | 8.1 | 0.41 | 10.4 | 3.34 |
| epigallocatechin gallate | 0.25 | 2.1 | 0.06 | 2.4 | 0.45 | 2.9 | 0.66 | 3.8 | 0.44 | 3.9 | 0.07 | 2.4 | 0.31 | 2.7 | 1.87 | 7.4 | 1.92 | 3.6 | 1.93 | 4.3 | 1.00 |
| epicatechin gallate | 0.025 | 8.0 | 1.48 | 8.4 | 3.60 | 9.3 | 2.67 | 23.0 | 4.91 | 23.1 | 5.15 | 25.9 | 8.71 | 19.5 | 3.56 | 24.0 | 1.83 | 28.7 | 7.89 | 50.3 | 14.11 |
| procyanidin B1 | 0.0125 | 147.1 | 15.25 | 180.1 | 54.86 | 109.4 | 15.24 | 263.9 | 73.12 | 236.7 | 7.98 | 335.1 | 36.84 | 167.8 | 72.37 | 407.4 | 97.80 | 168.0 | 23.45 | 421.4 | 123.10 |
| procyanidin B2 + B4 | 0.125 | 6.8 | 0.12 | 7.2 | 1.80 | 7.8 | 1.05 | 16.3 | 3.29 | 16.0 | 0.90 | 20.4 | 3.00 | 15.2 | 0.65 | 29.7 | 5.91 | 17.5 | 0.57 | 36.0 | 8.00 |
| procyanidin B3 (as B1) | 0.0125 | 76.8 | 1.38 | 99.2 | 32.19 | 64.5 | 13.30 | 180.5 | 31.26 | 156.6 | 16.92 | 207.7 | 36.15 | 119.5 | 2.33 | 295.5 | 71.83 | 139.5 | 35.74 | 373.3 | 138.36 |
| kaempferol | 0.0025 | 0.1 | 0.00 | 0.2 | 0.11 | 0.0 | 0.01 | 0.1 | 0.03 | 0.2 | 0.03 | 0.1 | 0.00 | 0.0 | 0.01 | 0.1 | 0.01 | 0.0 | 0.00 | 0.0 | 0.00 |
| quercetin | 0.05 | 1.3 | 0.06 | 1.9 | 0.10 | 1.1 | 0.19 | 2.8 | 0.47 | 2.3 | 0.06 | 3.3 | 0.85 | 2.9 | 0.69 | 3.5 | 0.23 | 1.1 | 0.15 | 2.2 | 0.24 |
| taxifolin | 0.0025 | 71.3 | 4.43 | 102.7 | 23.60 | 60.4 | 6.37 | 137.8 | 37.11 | 131.5 | 9.17 | 145.4 | 13.55 | 85.4 | 31.46 | 148.1 | 25.60 | 38.4 | 11.83 | 89.0 | 6.62 |

Supplemental Table S2.8. Continued

| Secondary metabolites | LOD(µg/g) | 12 hpi | | | | 24 hpi | | | | 48 hpi | | | | 72 hpi | | | | 96 hpi | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ctrl | | Trt | | Ctrl | | Trt | | Ctrl | | Trt | | Ctrl | | Trt | | Ctrl | | Trt | |
| | | mean | SE | Trt | SE | mean | SE | Trt | SE | mean | SE | Trt | SE | mean | SE | Trt | SE | mean | SE | Trt | SE |
| myricetin | 1.25 | 4.3 | 0.34 | 6.3 | 1.53 | 3.6 | 0.32 | 7.5 | 2.08 | 7.5 | 0.19 | 9.1 | 0.84 | 5.3 | 2.43 | 11.3 | 2.46 | 4.3 | 0.19 | 8.9 | 1.48 |
| quercetin-3-Rha | 0.005 | 12.6 | 1.26 | 16.2 | 2.78 | 7.6 | 0.29 | 16.3 | 5.38 | 17.6 | 1.11 | 19.4 | 1.01 | 13.6 | 5.98 | 20.4 | 2.78 | 3.4 | 0.02 | 7.7 | 0.05 |
| kaempferol-3-Glc | 0.0025 | 6.0 | 0.12 | 6.4 | 0.71 | 5.1 | 0.97 | 11.6 | 2.88 | 9.8 | 0.48 | 11.7 | 1.69 | 9.5 | 2.54 | 16.0 | 2.64 | 2.6 | 0.72 | 7.2 | 0.74 |
| quercetin-3-Glc+quercetin-3-Gal (as que-3-glc) | 0.0025 | 106.0 | 1.16 | 120.9 | 14.81 | 82.3 | 4.01 | 146.3 | 44.92 | 157.3 | 19.36 | 194.0 | 16.94 | 120.9 | 63.80 | 218.9 | 40.00 | 48.7 | 3.43 | 111.9 | 10.15 |
| isorhamnetin-3-Glc | 0.0025 | 2.2 | 0.13 | 2.8 | 0.29 | 1.8 | 0.14 | 3.7 | 0.97 | 3.7 | 0.07 | 5.3 | 0.55 | 2.8 | 0.28 | 4.6 | 0.71 | 1.3 | 0.29 | 3.4 | 0.76 |
| kaempferol-3-rutinoside | 0.005 | 2.7 | 0.36 | 4.6 | 1.08 | 1.2 | 0.07 | 2.6 | 0.61 | 5.0 | 0.67 | 3.3 | 0.22 | 1.8 | 1.17 | 3.1 | 0.55 | 0.5 | 0.15 | 1.2 | 0.11 |
| quercetin-3-Glc-Ara | 0.005 | 0.7 | 0.06 | 1.0 | 0.06 | 0.4 | 0.04 | 0.8 | 0.27 | 1.0 | 0.03 | 1.3 | 0.16 | 0.5 | 0.40 | 1.4 | 0.35 | 0.2 | 0.02 | 0.5 | 0.04 |
| rutin | 0.0025 | 24.0 | 1.06 | 31.6 | 1.98 | 12.9 | 0.86 | 29.2 | 9.80 | 35.1 | 2.88 | 40.1 | 2.07 | 20.1 | 13.54 | 40.4 | 8.30 | 6.9 | 0.07 | 16.3 | 1.31 |
| isorhamnetin-3-rutinoside | 0.005 | 0.4 | 0.08 | 1.0 | 0.33 | 0.2 | 0.01 | 0.7 | 0.13 | 1.1 | 0.10 | 0.6 | 0.00 | 0.3 | 0.03 | 0.6 | 0.11 | 0.1 | 0.02 | 0.2 | 0.01 |
| quercetin-3-glucuronide | 0.0125 | 294.9 | 14.05 | 476.6 | 66.20 | 247.4 | 22.56 | 542.5 | 72.97 | 508.6 | 28.91 | 551.0 | 52.89 | 324.9 | 180.12 | 694.9 | 151.05 | 197.6 | 20.52 | 475.7 | 72.83 |
| kaempferol-3-glucuronide | 0.005 | 13.8 | 0.98 | 23.1 | 4.78 | 13.9 | 2.50 | 30.1 | 8.14 | 29.1 | 1.94 | 23.0 | 3.27 | 17.7 | 10.60 | 17.2 | 0.19 | 5.9 | 1.94 | 18.0 | 0.63 |
| arbutin | 0.005 | 7.2 | 1.29 | 7.2 | 2.11 | 8.2 | 1.26 | 14.8 | 4.45 | 12.8 | 1.13 | 17.9 | 0.88 | 7.1 | 1.96 | 11.5 | 1.79 | 4.2 | 0.33 | 11.5 | 0.60 |
| *trans*-resveratrol | 0.005 | 0.3 | 0.02 | 2.2 | 0.88 | 0.4 | 0.13 | 1.6 | 0.42 | 0.3 | 0.04 | 1.4 | 0.50 | 0.2 | 0.58 | 0.7 | 0.21 | 0.0 | 0.01 | 1.7 | 0.81 |
| *cis*-resveratrol | 0.0025 | 0.0 | 0.00 | 0.0 | 0.00 | 0.0 | 0.00 | 0.0 | 0.00 | 0.0 | 0.00 | 0.0 | 0.01 | 0.0 | 0.00 | 0.0 | 0.00 | 0.0 | 0.00 | 0.0 | 0.00 |
| piceatannol | 0.0025 | 0.3 | 0.11 | 2.0 | 0.83 | 0.4 | 0.10 | 2.1 | 0.59 | 0.3 | 0.02 | 1.3 | 0.32 | 0.4 | 0.38 | 0.7 | 0.12 | 0.0 | 0.01 | 0.8 | 0.14 |
| *trans*-piceide | 0.0125 | 11.5 | 2.87 | 17.8 | 6.85 | 8.9 | 0.93 | 23.8 | 5.45 | 15.0 | 2.43 | 34.6 | 5.95 | 11.7 | 6.92 | 25.0 | 5.45 | 16.1 | 4.38 | 45.7 | 17.60 |
| *cis*-piceide | 0.0025 | 4.6 | 2.90 | 1.5 | 0.47 | 1.9 | 0.53 | 5.2 | 2.60 | 0.9 | 0.12 | 1.9 | 0.52 | 0.8 | 0.93 | 2.4 | 0.67 | 1.5 | 0.81 | 7.9 | 3.56 |
| astringin | 0.005 | 5.8 | 0.65 | 14.9 | 1.84 | 17.6 | 1.76 | 31.0 | 6.79 | 23.0 | 1.13 | 28.0 | 5.48 | 17.7 | 0.79 | 21.6 | 1.58 | 4.7 | 0.06 | 19.5 | 7.24 |
| isorhapontin | 0.005 | 0.3 | 0.03 | 0.5 | 0.02 | 0.8 | 0.06 | 0.7 | 0.29 | 0.9 | 0.07 | 0.8 | 0.11 | 0.6 | 0.06 | 0.6 | 0.00 | 0.3 | 0.06 | 0.9 | 0.05 |
| *trans*-ε-viniferin | 0.005 | 0.1 | 0.02 | 0.9 | 0.34 | 0.0 | 0.01 | 8.7 | 1.62 | 0.3 | 0.14 | 8.0 | 0.95 | 0.1 | 0.02 | 3.8 | 1.52 | 0.0 | 0.00 | 13.4 | 4.05 |
| *cis+trans*-o-viniferin | 0.0125 | 0.0 | 0.00 | 0.5 | 0.14 | 0.0 | 0.01 | 3.5 | 0.66 | 0.1 | 0.02 | 2.9 | 0.49 | 0.1 | 0.01 | 1.1 | 0.42 | 0.0 | 0.00 | 5.7 | 1.01 |

Supplemental Table S2.8. Continued

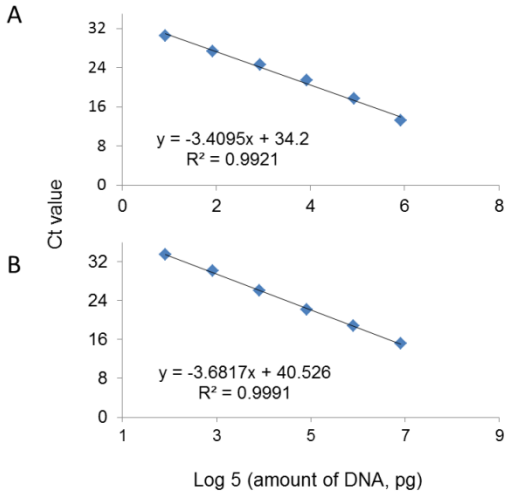
| Secondary metabolites | LOD(µg/g) | 12 hpi | | | | 24 hpi | | | | 48 hpi | | | | 72 hpi | | | | 96 hpi | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ctrl | | Trt | | Ctrl | | Trt | | Ctrl | | Trt | | Ctrl | | Trt | | Ctrl | | Trt | |
| | | mean | SE | Trt | SE | mean | SE | Trt | SE | mean | SE | Trt | SE | mean | SE | Trt | SE | mean | SE | Trt | SE |
| caffeic acid+catechin condensation | 0.05 | 6.3 | 0.10 | 9.6 | 1.91 | 8.8 | 0.44 | 19.3 | 5.67 | 18.2 | 0.46 | 17.2 | 2.84 | 22.1 | 3.60 | 10.2 | 4.85 | 5.6 | 0.53 | 15.0 | 3.39 |
| pallidol | 0.005 | 1.0 | 0.20 | 6.3 | 0.42 | 1.5 | 0.23 | 29.3 | 8.60 | 2.4 | 0.39 | 18.4 | 2.16 | 2.2 | 0.58 | 12.5 | 4.19 | 0.4 | 0.05 | 20.3 | 3.73 |
| ampelopsin D+quadrangularin A | 0.025 | 0.2 | 0.03 | 5.3 | 0.78 | 0.3 | 0.13 | 32.0 | 6.28 | 0.5 | 0.23 | 19.6 | 1.83 | 0.0 | 0.00 | 15.7 | 6.41 | 0.1 | 0.02 | 18.1 | 1.86 |
| α-viniferin | 0.0125 | 0.4 | 0.12 | 2.8 | 1.58 | 0.1 | 0.03 | 137.5 | 50.16 | 1.1 | 0.63 | 90.9 | 3.62 | 0.2 | 0.03 | 96.9 | 39.49 | 0.1 | 0.06 | 151.8 | 38.50 |
| E-*cis*-miyabenol | 0.0125 | 0.2 | 0.02 | 1.5 | 0.20 | 0.0 | 0.02 | 35.5 | 9.02 | 0.3 | 0.11 | 15.8 | 2.07 | 0.2 | 0.06 | 15.8 | 6.35 | 0.0 | 0.01 | 53.0 | 4.95 |
| Z-miyabenol C | 0.05 | 0.1 | 0.03 | 1.2 | 0.25 | 0.1 | 0.01 | 33.1 | 9.56 | 0.3 | 0.22 | 14.7 | 1.60 | 0.1 | 0.03 | 16.0 | 6.51 | 0.1 | 0.00 | 46.4 | 6.95 |
| isohopeaphenol | 0.0125 | 0.1 | 0.03 | 0.2 | 0.11 | 0.0 | 0.01 | 13.9 | 7.24 | 0.2 | 0.10 | 17.6 | 0.45 | 0.1 | 0.01 | 11.1 | 4.49 | 0.0 | 0.00 | 20.9 | 1.99 |
| sinapaldehyde | 0.00125 | 0.1 | 0.01 | 0.1 | 0.06 | 0.0 | 0.01 | 0.0 | 0.01 | 0.1 | 0.02 | 0.1 | 0.01 | 0.1 | 0.03 | 0.1 | 0.01 | 0.0 | 0.00 | 0.2 | 0.06 |
| 5-hydroxyferulic acid | 0.00125 | 2.3 | 0.34 | 2.3 | 0.29 | 1.3 | 0.20 | 1.6 | 0.34 | 2.6 | 0.37 | 2.5 | 0.10 | 2.3 | 0.66 | 1.9 | 0.28 | 1.2 | 0.02 | 1.5 | 0.10 |
| L-phenylalanine | 0.00125 | 42.7 | 0.12 | 62.6 | 3.90 | 76.8 | 7.01 | 131.2 | 16.34 | 129.8 | 0.87 | 99.8 | 13.42 | 81.5 | 3.95 | 110.5 | 2.46 | 82.8 | 3.46 | 73.7 | 5.35 |
| cinnamic acid | 0.0025 | 0.0 | 0.02 | 0.0 | 0.00 | 0.0 | 0.01 | 0.1 | 0.01 | 0.0 | 0.01 | 0.0 | 0.00 | 0.1 | 0.00 | 0.1 | 0.00 | 0.1 | 0.01 | 0.1 | 0.01 |
| *p*-coumaric acid | 0.0025 | 1.2 | 0.32 | 1.3 | 0.47 | 0.6 | 0.22 | 0.5 | 0.14 | 0.6 | 0.11 | 0.8 | 0.05 | 0.8 | 0.52 | 0.5 | 0.23 | 0.2 | 0.01 | 0.2 | 0.04 |
| caffeic acid | 0.00125 | 0.7 | 0.05 | 0.6 | 0.12 | 0.4 | 0.03 | 0.3 | 0.08 | 0.4 | 0.04 | 0.5 | 0.02 | 0.4 | 0.09 | 0.4 | 0.08 | 0.5 | 0.02 | 0.4 | 0.03 |
| ferulic acid | 0.00125 | 1.3 | 0.57 | 2.7 | 0.50 | 0.7 | 0.19 | 0.6 | 0.13 | 1.5 | 0.19 | 1.0 | 0.25 | 0.5 | 0.07 | 0.8 | 0.47 | 0.1 | 0.02 | 0.1 | 0.08 |
| coniferyl alcohol | 0.00125 | 0.1 | 0.02 | 0.1 | 0.01 | 0.1 | 0.05 | 0.2 | 0.01 | 0.1 | 0.04 | 0.2 | 0.05 | 0.2 | 0.08 | 0.3 | 0.07 | 0.2 | 0.03 | 0.4 | 0.07 |

Supplemental Table S2.9. *B. cinerea* genes expressed *in planta*. (Provided as excel file)

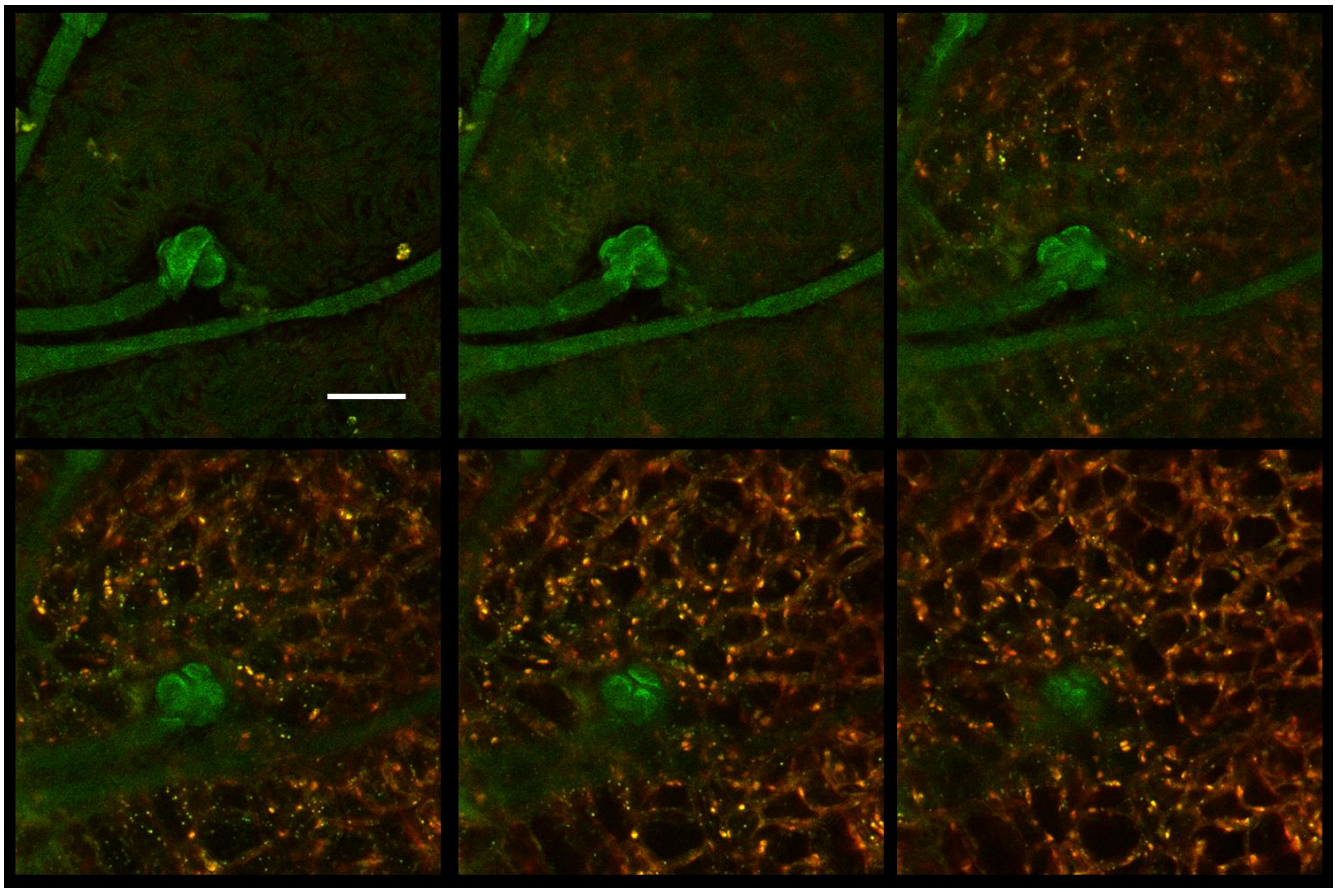
Supplemental Table S2.10. Gene ontology (GO) annotation of *B. cinerea* genes expressed in planta. (Provided as excel file)

## 2.8.2. Supplemtnetal figures

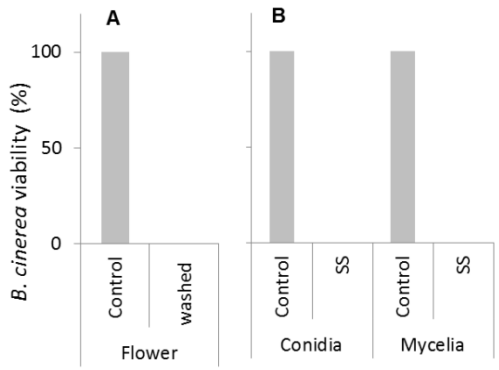

Supplemental Figure S2.1. Optical microscopy images of *B. cinerea* conidia cultured for 12 hours in PDB. Right panel shows a higher magnification view. This growth stage was used as control for the fungal transcriptomic study. C, conidia; Gt, germ tube; the bar represents 10 µm.




Supplemental Figure S2.2. Standard curves used for grapevine and *B. cinerea* genomic DNA quantification. Curves were generated by amplifying the *Bc3* (ribosomal IGS spacer) (A) and the *VvRS I* (resveratrol synthase gene I) (B) genes, in a 5-fold serially diluted genomic DNA of *B. cinerea* and grapevine, respectively. The standard curves show good linear relationship ($R^2$ = 0.99) between the log5 value of the starting DNA concentration and the threshold cycle (Ct), both in the fungus and the plant.



Supplemental Figure S2.3. Z stack images of GFP-labeled B05.10 *Botrytis* infecting a grapevine flower. The confocal microscope image shows six layers of a Z stack with a pass of 1 µm each. The figure indicates that the *Botrytis* appressorium penetrates only the first epidermal layers within 24 hpi. The white bar represents 15 µm.

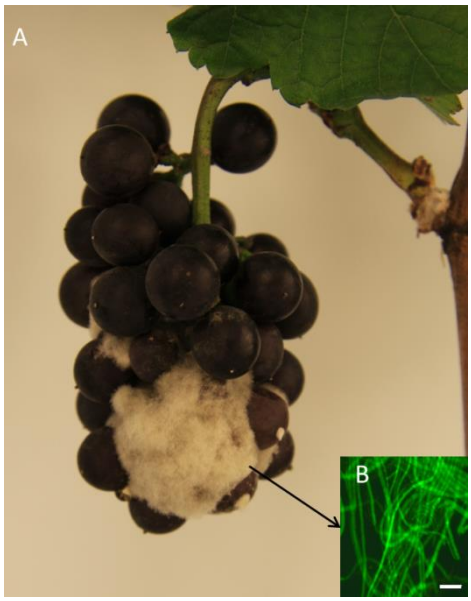



Supplemental Figure S2.4. Effects of washing within 6 hpi (A) and surface sterilization (SS) (B) on the viability of *B. cinerea*.

Description of Supporting Information Fig. S4

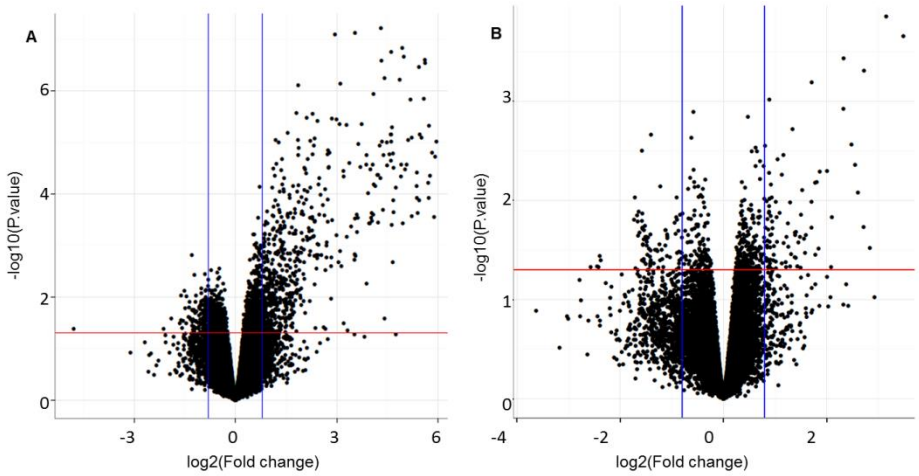
A preliminary study was conducted to know the effect of grapevine flowers washing and surface sterilization (SS) on the viability of *B. cinerea*. Grapevine flowers were collected from fruiting cuttings at cap-off stage, 16 flowers from each of 3 fruiting cuttings (replicates). Flowers were then inoculated with 300 conidia of GFP-labelled B05.10 strain and incubated in Petri dishes with moistened paper towel for 6 hours, a time span within which the conidium will not germinate. The inoculated flowers were further incubated for one week on selective media (PDA with70 µg/ml Hygromycin B), before or after washing. Washing was carried out as described in Materials and Methods section. As shown in 2A, mycelial growth was observed on 100 % of the flowers that were plated out without washing, but on none of the washed flowers, indicating that washing was effective in removing ungerminated conidia.

To check the effect of surface sterilization on the viability of *B. cinerea*, conidia and mycelia (harvested from Petri dishes of the same strain mentioned above) were treated as described in Materials and Methods and then allowed to grow on selective media. As shown in of Supporting Information Fig. S4B, conidia and mycelia did not grow after sterilization.

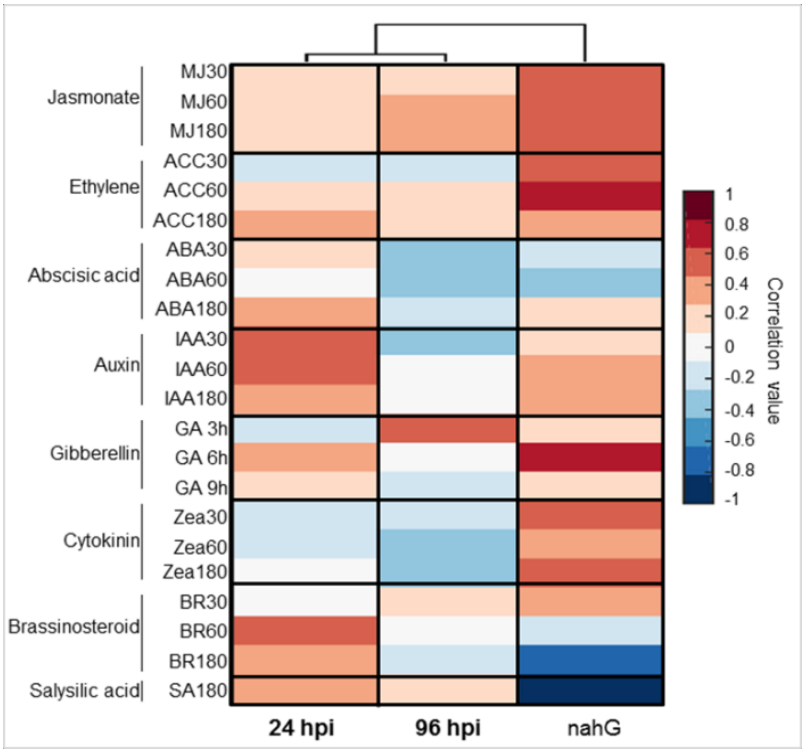



Supplemental Figure S2.5. Egression of *B. cinerea* at ripening (A), inoculated at full cap-fall stage, and fluorescence of mass of *B. cinerea* mycelia isolated from the rotting bunch (B). The white bar represents 25 µm.

Supplemental Figure S2.6. *B. cinerea* egression test on immature berries. Immature berries at pepper-corn stage, which were inoculated with *B. cinerea* at flower cup-off stage, were bagged for 2 weeks. No egression was observed during the period.




Supplemental Figure S2.7. Volcano plots of grapevine gene expression values at 24 hpi (A) and 96 hpi (B). Genes in the left top rectangle [log 10(*P*. value) > 1.12 and log2(fold change) < -0.585] were selected as downregulated; whereas, genes in the right top rectangle were selected as upregulated [log 10(*P*. value) > 1.12 and log2(fold change) > 0.585] .




Supplemental Figure S2.8. Hormonal Signatures based on transcriptomic data. HORMONOMETER software was used to compare gene expression data of our experiment, as query experiment, with data sets of hormone responses. Transcriptomes of *Arabidopsis* mutant, *nahG*, with known alteration in salicylic acid level was used as control. The scale bar shows the correlation between the query transcriptome and the hormone-treated indexed data set.

Description of Supporting Information Fig. S8

HORMONOMETER software (Volodarsky *et al.* 2009) was used to evaluate hormonal responses in grapevine inflorescence upon *B. cinerea* inoculation. Since the software accepts only *Arabidopsis thaliana* gene IDs as input, *Arabidopsis* orthologs of grapevine genes provided by Grimplet *et al.* (2012) were taken. Out of the expressed grapevine genes in our experiment, 10507 had *Arabidopsis* orthologs, and hence were used to investigate the hormonal profiles of grape flower due to *B. cinerea* inoculation. Gene expression data of *Arabidopsis* mutant with reduced salicylic acid levels, *nahG*, (GEO, GSE5727) was also included to validate the set of genes used in the analysis.

As depicted in Suppportign Information Fig. S8, the HORMONOMETER profile of *Botrytis*-inoculated flowers showed positive correlations with salicylic acid, brassinosteroid, gibberellin, auxin, abscisic acid (ABA), and ethylene treatments at 24 hpi and with jasmonate treatment at 96 hpi. Whereas, a negative correlation was observed with ABA and cytokinin treatments at 96 hpi; the latter one also showed similar trend at 24 hpi. Although the role of cytokinin in plant defense is largely unknown, the profile shown by the HORMONOMETER could be related to the cell cycle network enriched in the downregulated genes shown in Table 2. With regard to ABA, its role in plant defense appears to vary among different types of plant-pathogen interactions (Bari and Jones, 2009). In tomato, the induction of ABA biosynthetic genes was reported to facilitate *B. cinerea* colonization by inducing senescence, and on the other hand, ABA-deficient *sitiens* mutant was shown to be more resistant to the pathogen (Audenaert *et al.* 2002; Blanco-Ulate *et al.* 2013).

# 3. INTERACTION BETWEEN GRAPE BERRY AND *B. cinerea* DURING QUIESCENT AND EGRESSED INFECTIONS

## 3.1. Abstract

*Botrytis cinerea* is an important pathogen in vineyards where primary infections are mostly initiated by airborne conidia from overwintered sources around bloom. The fungus often remains quiescent from bloom until maturity and egresses at ripe where it causes bunch rot. Studying the interaction between the fungus and the host during quiescent and egressed infection stages help understand the cross talk between the two organisms. Therefore, flowers from fruiting cuttings of the cv. Pinot noir were inoculated with GFP labelled *B. cinerea* at at full cap-off stage, and molecular analyses were carried out at 4 weeks post inoculation (wpi), fungus quiescent state, on hard-green berries and at 12 wpi, fungus pre-egression and egression states, on ripe berries. During the quiescent state, the expressed fungal transcriptome highlighted that the fungus was undergoing basal metabolic activities besides remodeling its cell wall to evade plant chitinases. Hard-green berries responded by differentially regulating genes encoding for different PR proteins and genes involved in monolignol, flavonoid and stilbenoid biosynthesis pathways which kept the pathogen quiescent. At 12 wpi, the transcripts of *B. cinerea* in the pre-egressed samples showed that virulence-related genes were expressed, suggesting infection process was initiated. The egressed *B. cinerea* expressed almost all virulence and growth related genes that enabled the pathogen to colonize the berries. In response to egression, ripe berries reprogramed different defense responses, though futile. Our results indicated that hard-green berries defense program was capable to contain *B. cinerea*; however, ripening associated fruit's cell wall self-disassembly together with high humidity created the opportunity for the fungus to egress and cause bunch rots.

## 3.2. Introduction

*Botrytis cinerea* is responsible for a significant economic damage in vineyards by causing bunch rot. The disease is mostly observed on ripe berries, following rainfalls or long periods of high humidity close to harvest, and develops into gray mold. Primary infections are usually initiated by airborne conidia from overwintered sources (Nair et al., 1995; Elmer and Michailides, 2004), which mostly happens at bloom leading to quiescent infection (McClellan and Hewitt, 1973; Keller et al., 2003; Pezet et al., 2003b). Quiescent infection is an interesting phenomenon in *B. cinerea*-plant interaction where the pathogen spends prolonged time in the host tissue asymptomatically, without being aggressive (Williamson et al., 1987; McNicol and Williamson, 1989; Coertze and Holz, 2002; Shaw et al., 2016). Besides *Botrytis*, several species of fungal pathogens belonging to the genera *Alternaria*, *Botryospheria*, *Colletotrichum*, *Lasiodiplodia*, *Monilinia, Phomopsis,* and *Sclerotinia*, have been reported to pass through a quiescent state in the cuticular wax or intercellular space of their hosts until conditions favor egression (Reviewed by Prusky et al., 2013).

In grapevine, *B. cinerea* egression, causing bunch rot, was observed at ripening from the artificial inoculation made at bloom, where the pathogen stayed quiescent in the developmental stages between fruitlets to maturity (see in Chapter 2). What drives and keeps *B. cinerea* into quiescence until berry ripening is not fully known, but preformed and induced defense mechanisms, including immature berries skin features such as polyphenols in the berry skin cell wall and the thickness of epidermal cell layer complex, have been proposed as part of the ontogenic resistance to *B. cinerea* (Deytieux-Belleau et al., 2009; Goetz et al., 1999; Keller et al., 2003). Recently, it has been shown that upon contact with the grapevine flower, *B. cinerea* induces genes encoding known virulence factors (like *BcBOA6*, *BcBOT*, *BcPG2*, and *BcSOD1*) and proteins contributing to the infection program (like *BcOAH*, *BcXYN11A*, and *BcGST1*) to cause disease. However, no visible disease progress was observed despite the confirmed presence of the pathogen on the immature berries (Chapter 2). As a response to the infection attempt, grapevine flowers react by reprogramming the expression of genes encoding antimicrobial proteins (mainly PR-proteins), monolignol biosynthesis (*VvPAL*, *VvCOMT*, *VvCCoAMT* and *VvCAD* ), stilbenoids (*VvSTS*), and prompting oxidative burst (*VvGLP3*). The conjugated actions of these induced defense responses contribute for putting *B. cinerea* into quiescence (Chapter 2). In addition, the involvement of the salicylic acid (SA) dependent defense pathway together with the accumulation of ROS and the

activation of stilbene and lignin biosynthesis was reported as the possible reasons of *B. cinerea* progress arrest in berries at véraison while ripe berries were fully susceptible to the pathogen (Kelloniemi et al., 2015).

The transition from a quiescent to an active infection mostly occurs during senescence/fruit ripening. Physiological and biochemical changes that occur in the host during ripening, together with favorable climatic conditions at ripening are suggested to take a part in triggering the transition (Prusky, 1996; Barnes and Shaw, 2002; Prusky et al., 2013). Cell wall loosening and appearance of disassembled cell wall substrates (Cantu et al., 2008), decrease in preformed and inducible host defense responses and change in hormonal balance and pH (Prusky, 1996; Prusky et al., 2013) are the major changes happening in the processes of berry ripening that could enhance the outgrowth of a quiescent necrotrophic pathogen. Egression impairs product quantity, quality, and appearance.

Global expression profiling of both the pathogen and the host at quiescent and egression stages of the infection enables to gain insight into the signaling, the metabolic pathways, the transcriptional control, and the defense responses involve in the cross talk. Hence, the objective of this study was to understand the molecular mechanisms associated with *B. cinerea* and grapevine berry interaction during quiescent and egression stages. Here we report the simultaneous transcriptome and secondary metabolite analyses of the two organisms at berry hard-green and ripe stages, after host inoculation with *B. cinerea* conidial at full cap-off stage (EL25/26).

## 3.3. Materials and methods

### 3.3.1. Fungal isolate, plant material and inoculation

A genetically transformed *Botrytis cinerea* strain, B05.10, expressing a green fluorescent protein (GFP), was used due to its ability to grow on selective medium (potato dextrose agar [PDA] + 70 µg/ml Hygromycin B) besides giving a green fluorescent signal when observed under fluorescence microscopy. Grapevine fruiting cuttings were used from cv. Pinot Noir. Winter woody cuttings were collected and grown to raise flowers as described in (Chapter 2).

Flowers at full cap-fall stage (EL25/26, according to Eichorn and Lorenz [1977]) were inoculated by placing a 1.5 µl droplet of either conidia solution of GFP-labeled B05.10 ($2 * 10^5$ $ml^{-1}$) or distilled water (mock inoculation) close to the receptacle area. Conidia were obtained from

*Botrytis* grown on PDA at 25 °C for 10 days, and the concentration was determined using a hemacytometer under light microscope. Inoculation was made on three biological replicates, considering the inflorescence from a fruiting cutting as one biological replicate. After inoculation, the whole pot was immediately bagged in a clear plastic bag sprayed with water, for 24 h, in order to ensure high humidity around the inoculated inflorescence, which is an essential factor for conidial germination. Inoculated inflorescences were regularly inspected for gray mold growth until fruit ripening. At full coloring (approximately 10 weeks post inoculation, wpi), bunches were bagged for two weeks with plastic bags, to create favorable humidity for *B. cinerea* to egress.

Samples were collected at two time points, at 4 wpi, hard green berry, and at 12 wpi, from ripe clusters where *Botrytis* egression was evident on a subset of berries. For the latter time point, two kinds of samples were collected: berries with visible egressed *Botrytis* and berries without visible *Botrytis* sign. Samples without visible *Botrytis* sign are hereafter called berries with "pre-egressed" *Botrytis*. Samples were snap frozen in liquid nitrogen and stored at -80 °C until use.

### 3.3.2. RNA extraction, qPCR and RNA-seq

Extraction of RNA, synthesis of cDNA, and quantitative polymerase chain reaction (qPCR) assay were carried out as described in (Chapter 2). For qPCR assay, each amplification reaction was run in triplicate, and *VvACT* and *VvGAPDH*, and *BcRPL5* and *BcTUBA* genes were selected using GeNORM (Vandesompele et al., 2002) as reference for grapevine and *B. cinerea*, respectively, for normalization. Amplification efficiencies of each primer pair were calculated with LinReg software (Ruijter et al., 2009). The obtained amplification efficiency was used to calculate the relative quantity (RQ) and normalized relative quantity (NRQ) according to Hellemans et al. (2007). All primers and corresponding gene identifiers are listed in Supplemental Table S3.1.

Single-end reads of 100 bp long sequences were obtained for each sample using a Next Generation Sequencing Platform HiSeq 1500 (Illumina, San Diego, CA). Approximately 20 million strand-specific sequences, except for pre-egressed samples (above 45 million), was obtained. For pre-egressed samples, the sequence depth was doubled in order to obtain more reads of *Botrytis* origin. The quality of the reads was checked using FastQC (version 0.11.2) software and pre-processed by cutadapt (version 1.8.1; Martin, [2011]) for adapter. Genome

assemblies of grapevine (12Xv1, http://genomes.cribi.unipd.it/) and *B.cinerea* (strain B05.10) (http://fungi.ensembl.org) were used as reference sequences. The alignment was made by Subread aligner (Liao, et al., 2013) and raw read counts were extracted using the featureCount read summarization program (Liao, et al., 2014).

The RNA sequences of *B. cinerea* (B05.10), from the PDB cultured conidia (used in Chapter 2) were also used in this study as a control for differential expression analysis for the *in planta Botrytis* transcripts.

### 3.3.3. Secondary metabolites extraction and analysis

Extraction of polyphenol and Ultra High Performance Liquid Chromatography - Diode Array Detection - Mass Spectrometry (UHPLC-DAD-MS) analysis were carried out as described in (Chapter 2). For 4 wpi, the samples used for polyphenol and RNA extraction were independent.

### 3.3.4. Statistical analysis

Statistical analyses of the qPCR results were made after log2(NRQ) transformation (Rieu and Powers, 2009). Statistical significance was calculated by Tukey's Honestly Significant Difference test or an unpaired heteroscedastic Student's *t* test, considering each technical replicate as an individual sample.

Differential expression analysis was performed after precision weight was given, by the voom method (Law, et al., 2014), for each observation that was fed into the limma empirical Bayes analysis pipeline (Smyth, 2004). Two-sample t-test was used for transcripts of grapevine at 4 wpi (mock inoculated vs *Botrytis* inoculated) and *B. cinerea* at egression (PDB-cultured *Botrytis* vs egressed *Botrytis*), whereas one-way ANOVA for grapevine transcripts at 12 wpi (mock inoculated vs pre-egressed *Botrytis* vs egressed *Botrytis*). Genes were considered differentially expressed (DE) if they fulfill a $p$-value $< 0.01$ and an absolute fold change of $\geq 2.0$.

Principal component analysis (PCA) was performed using prcomp package in R on precision weight given counts. *K*-means clustering of differentially expressed genes based on fold change values (using cosine distance) and precision weight given counts (using Euclidean distance) was performed using akmeans package in R.

### 3.3.5. Functional classification based on Gene Ontology, VitisNet, and MapMan

DE genes were subjected to enrichment analyses using: i) VitisNet annotation within the VESPUCCI grapevine gene expression compendium (http://vespucci.colombos.fmach.it) (Grimplet et al., 2012; Moretto et al., 2016), *p*-value < 0.01; ii) customized GO annotation and annotated reference, taken from CRIBI annotation (http://www.cribi.unipd.it/), using AgriGO analysis tool (http://bioinfo.cau.edu.cn/agriGO/analysis.php; Du et al., 2010). Enriched GO terms (FDR < 0.01) were visualized using the 'Reduce + Visualize Gene Ontology' (REViGO) webserver (http://revigo.irb.hr; Supek et al., 2011). Additionally, the differentially expressed genes were visualized in the context of biotic stress pathway using the GrapeGen 12Xv1 annotations version (Lijavetzky et al., 2012) with the help of MapMan tool (Thimm et al., 2004)


Figure 3.1. *Botrytis cinerea* infected grapevine flowers and their growth until maturity. **A**, Flowers 24 h after inoculation with GFP-labelled B05.10 at full cap-fall stage (EL25/26). **B**, Healthy looking, asymptomatic, hard-green berry at 4 weeks post inoculation (wpi) **C**, Egression of *B. cinerea* at ripening (12 wpi). **D**, Fluorescence of mass of mycelia isolated from the outgrown *B. cinerea*; white bar represents 50 µm.

## 3.4. Results

### 3.4.1. *Botrytis cinerea* inoculation of grapevine

Grapevine flowers were inoculated with a GFP-labeled B05.10 strain at full cap-off stage by placing 300 conidia around the receptacle area, and the infection was monitored until ripening, for 12 weeks (Figure 3.1). No visible symptom or sign of the fungus was observed until full coloring, though appressorium assisted penetration of floral epidermis at 24 hours post inoculation has been observed and described in detain in Chapter 2. The proportion berries still carrying *B. cinerea* at hard-green berry stage (4 wpi; Figure 3.1C) derived from flower inoculation was checked by plating out on selective media (Figure 3.2). *B. cinerea* was present on 80 % of the asymptomatic berries quiescently when samples were washed or not with water; the proportion dropped to 40 % when berries were surface sterilized, suggesting that the fungus mostly resides in the first few outer epidermal cell layers. At 10 wpi, full color change, bunches were bagged with plastic bags, to increase humidity and favor *B. cinerea* growth. Two weeks later, egression of *B. cinerea* was observed (Figure 3.1C), and cross checking the strain using fluorescence microscope, on mass of mycelia taken from the rotting bunch, confirmed that the strain was the GFP-labeled B05.10 inoculated at cap-off stage (Figure 3.1D). Egression was observed on about 40 % (39 ± 9 %) of the inoculated berries.

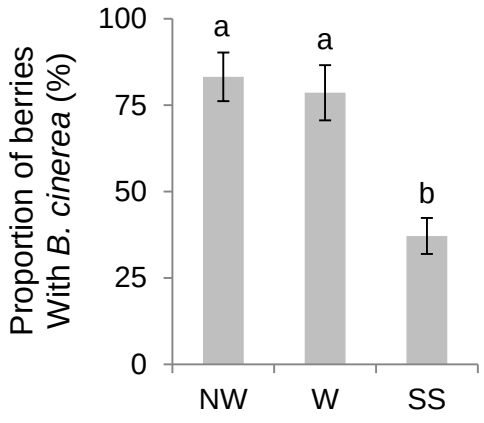



Figure 3.2. Proportion of berries with *B. cinerea* at 4 wpi, following flower inoculation with the fungus, as determined by plating out. Plating out was made on selective media (PDA with Hygromycin B, 70 µg/ml) to check the presence of quiescent *B. cinerea* before (NW) or after washing (W), or after surface sterilization (SS). Mean proportion of berries (8-10 berries from each of 6 biological replicates) showing GFP-labeled B05.10 growth on the selective media. Error bars indicate standard error. Mean proportions followed by a common letter are significantly not different, according to Tukey's Honestly Significant Difference test ($P \leq 0.05$), using one way ANOVA.

### 3.4.2. Transcriptional profiling of grapevine berry responses to *B. cinerea*

Hard green (4 wpi) and ripe berries (12 wpi), which were mock- or *Botrytis*-inoculated at cap-off stage, were harvested in 3 biological replicates for dual (plant and fungus) transcriptome analysis using the RNA-seq method. The fraction of reads from *Botrytis*- and mock-inoculated samples

mapped to the *V. vinifera* reference genome ranged from 13 to 88 %, the smaller proportion being from samples with egressed *B. cinerea*. However, in the case of *Botrytis*-inoculated samples mapped to *B. cinerea* reference genome, the fraction of mapped reads was below 1 % for the 4 wpi and pre-egressed samples, and up to 68 % for egressed *B. cinerea* samples (Supplemental Table S3.2).

The biological variability of all the samples was assessed using principal component analysis (PCA) on the gene expression data. Concerning grapevine data, samples were largely separated by growth stage along the first principal component, but within each growth stage most of the variation in gene expression was explained by the infection process (Figure 3.3A). With regard to *B. cinerea*, PDB grown and egressed samples were compared by PCA, highlighting the amazing difference in gene expression between the egression stage on berries at ripening and the growth in liquid PDB medium (Figure 3.3B)..

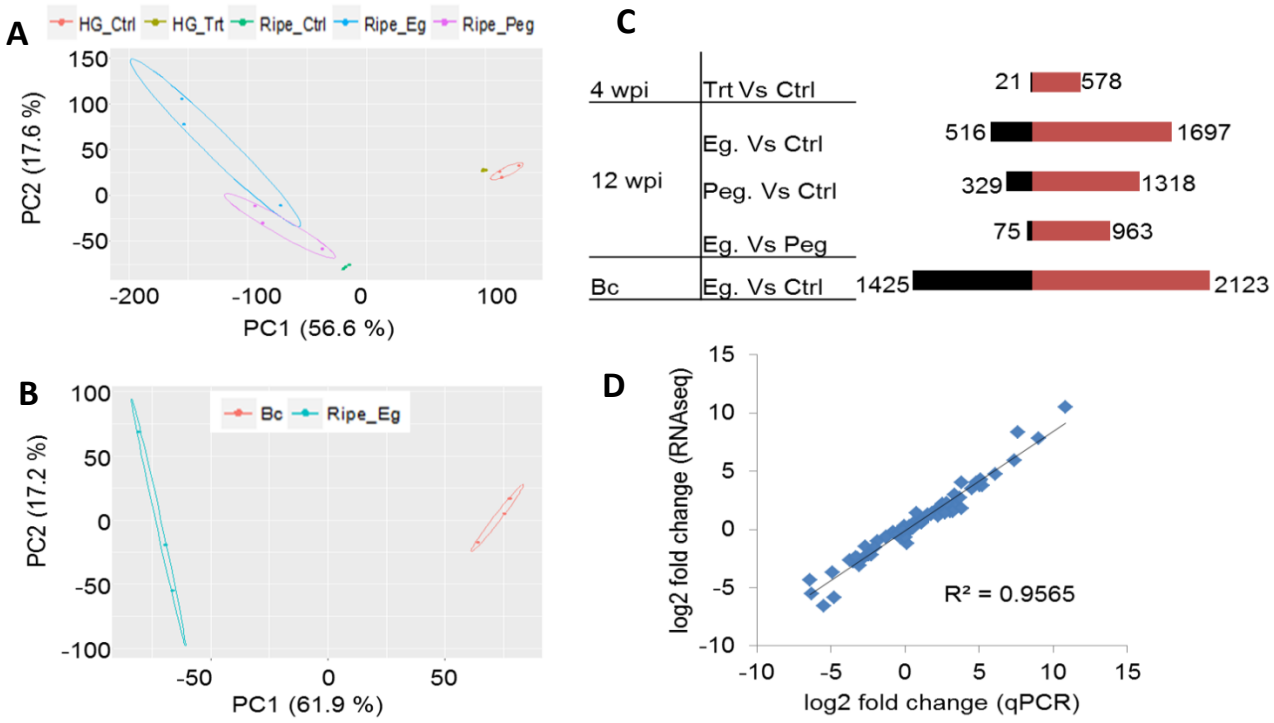


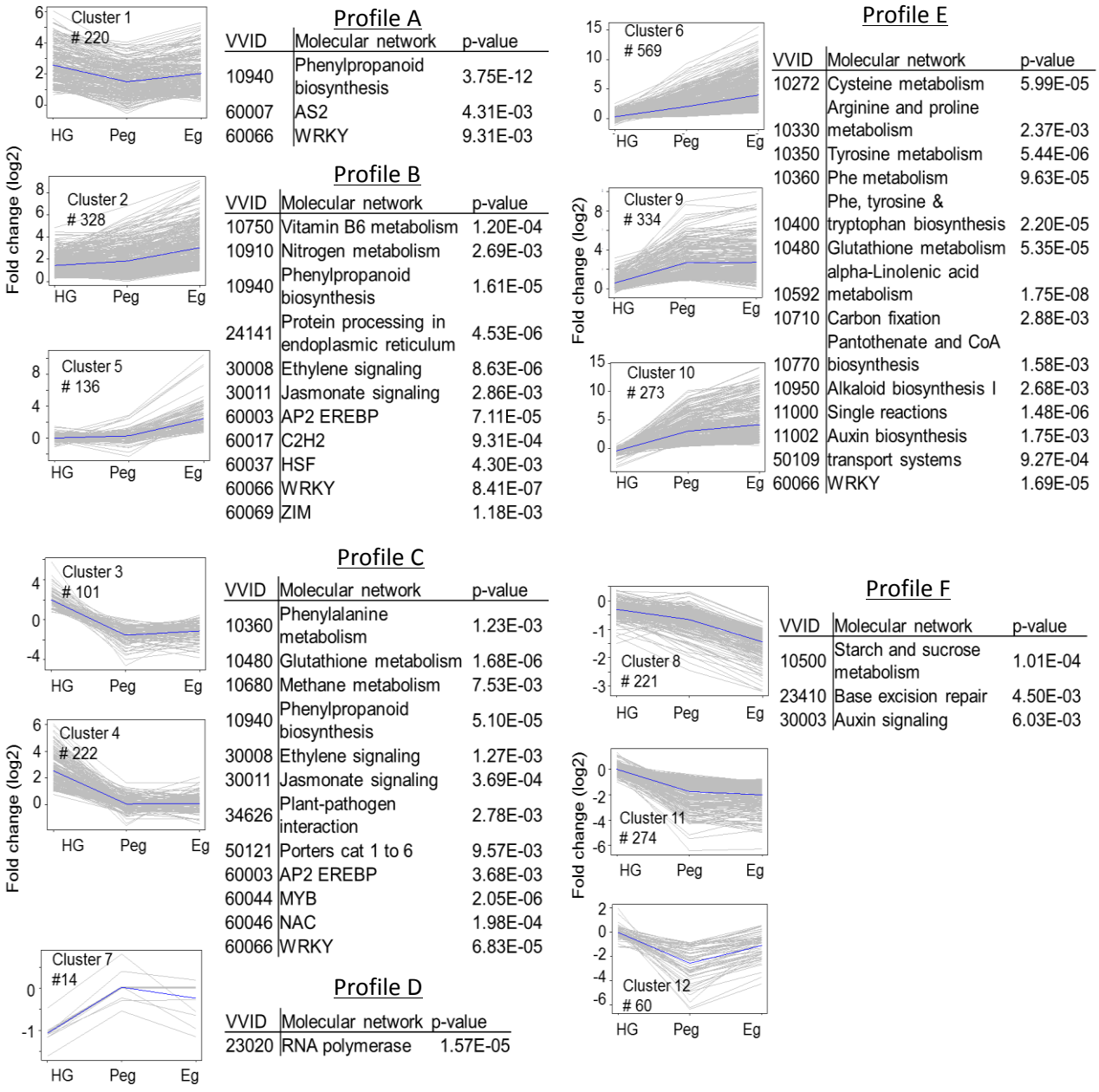

Figure 3.3. Global evaluation of the RNA-seq data and of the differentially expressed (DE) genes. PCA displaying the biological variations among samples,for grapevine genes (**A**) and *B. cinerea* genes (**B**). Ctrl, mock inoculated; Trt, *B. cinerea* inoculated; Bc, *Botrytis cinerea;* HG, hard-green berry; Eg, *B. cinerea* egression state; Peg, *B. cinerea pre*-egression state. Raw count data were used after precision weight was calculated by the voom method (Law et al., 2014). **C**, Number of DE genes ($P < 0.01$, absolute fold change > 2.0) upon *B. cinerea* infection at 4 weeks post inoculation (wpi) and 12 wpi; upregulated genes (red) and downregulated genes (black). Bc, *Botrytis cinerea.* **D**, Correlation of gene expression values obtained by RNA-seq and qPCR. Relative expression levels were calculated for 18 *Vitis* genes and an $R^2$ value of 0.96 was obtained comparing the results obtained with the two techniques.

Differential expression of genes was computed between *Botrytis*- and mock-inoculated berries at hard green stage (4 wpi) and at ripening (12 wpi) imposing a *p*-value < 0.01 and an absolute fold change > 2 (Supplemental Figure S3.1). At 4 wpi, 599 genes of grapevine were differentially expressed (DE) due to *B. cinerea* infection (Supplemental Table S3.3), whereas the number increased to 2,311 at ripening (Figure 3.3C and Supplemental Table S3.4). Only 158 genes were common between the two stages, suggesting an apparent different reaction of the host. For *B. cinerea* during egression, as compared to PDB cultured *Borytis*, there were 3,548 DE genes (Figure 3.3C and Supplemental Table S3.5). However, for 4 wpi and pre-egressed samples, due to limited amount of fungal RNA in the samples (Supplemental Table S3.6 and S3.7), it was not possible to gain insight into the differential expression of transcriptome of the fungus in the host using the RNA-seq results. To overcome the technical problem, qPCR assay was used to study the expression profile of selected genes.

The gene expression values obtained from RNA-seq were validated using qPCR assay. To this end, the expression of 18 grapevine genes (Supplemental Table S3.8) having different expression profile (differentially expressed or not) from RNA-seq were analyzed and a strong correlation ($R^2$ = 0.96) was observed between the results obtained from the two teqniques (Figure 3.3D).

The total DE genes of the grapevine berries (2,752), considering both hard-green and ripe stages, were clustered into 12 distinct groups based on their expression pattern (Figure 3.4). Based on the expression trend of the DE genes, the clusters fell into 6 major expression profiles: profile A consisted only cluster 1, the upregulation extent of the majority of the 220 genes were higher at hard green stage as compared to ripe stage; profile B consisted cluster 2 and 5, the majority of the 464 genes was slightly upregulated or not affected at quiescence and pre-egression stage but highly upregulated during egression of *B. cinerea*; profile C consisted cluster 3 and 4, where majority of the 323 genes were upregulated at hard green stage and downregulated or not affected at ripe; profile D consisted cluster 7, having only 14 genes downregulated more at hard green than at ripe stage; profile E consisted cluster 6, 9 and 10, and majority of the 1176 genes were upregulated during ripening only; and profile F consisted cluster 8, 11 and 12, where the expression of majority of the 555 genes was not affected at hard green stage but downregulated during ripening. The molecular networks enrichment analysis of the gene set of each profile, based on VitisNet annotation, showed an abundance of transcripts in functional classes which are

usually affected by biotic stress. A considerable number of genes annotated for phenlpropanoid biosynthesis and transcriptional factors (TF) were represented in all expression profiles except for profile D and F. Signal transduction, such as ethylene and jasmonate signaling and plant-pathogen interaction, was represented in profile B and C. Several transcripts involved in amino acid metabolism, including glutathione metabolism, were represented in expression profile E.




**Profile A**

| VVID | Molecular network | p-value |
|---|---|---|
| 10940 | Phenylpropanoid biosynthesis | 3.75E-12 |
| 60007 | AS2 | 4.31E-03 |
| 60066 | WRKY | 9.31E-03 |

**Profile B**

| VVID | Molecular network | p-value |
|---|---|---|
| 10750 | Vitamin B6 metabolism | 1.20E-04 |
| 10910 | Nitrogen metabolism | 2.69E-03 |
| 10940 | Phenylpropanoid biosynthesis | 1.61E-05 |
| 24141 | Protein processing in endoplasmic reticulum | 4.53E-06 |
| 30008 | Ethylene signaling | 8.63E-06 |
| 30011 | Jasmonate signaling | 2.86E-03 |
| 60003 | AP2 EREBP | 7.11E-05 |
| 60017 | C2H2 | 9.31E-04 |
| 60037 | HSF | 4.30E-03 |
| 60066 | WRKY | 8.41E-07 |
| 60069 | ZIM | 1.18E-03 |

**Profile C**

| VVID | Molecular network | p-value |
|---|---|---|
| 10360 | Phenylalanine metabolism | 1.23E-03 |
| 10480 | Glutathione metabolism | 1.68E-06 |
| 10680 | Methane metabolism | 7.53E-03 |
| 10940 | Phenylpropanoid biosynthesis | 5.10E-05 |
| 30008 | Ethylene signaling | 1.27E-03 |
| 30011 | Jasmonate signaling | 3.69E-04 |
| 34626 | Plant-pathogen interaction | 2.78E-03 |
| 50121 | Porters cat 1 to 6 | 9.57E-03 |
| 60003 | AP2 EREBP | 3.68E-03 |
| 60044 | MYB | 2.05E-06 |
| 60046 | NAC | 1.98E-04 |
| 60066 | WRKY | 6.83E-05 |

**Profile D**

| VVID | Molecular network | p-value |
|---|---|---|
| 23020 | RNA polymerase | 1.57E-05 |

**Profile E**

| VVID | Molecular network | p-value |
|---|---|---|
| 10272 | Cysteine metabolism | 5.99E-05 |
| 10330 | Arginine and proline metabolism | 2.37E-03 |
| 10350 | Tyrosine metabolism | 5.44E-06 |
| 10360 | Phe metabolism | 9.63E-05 |
| 10400 | Phe, tyrosine & tryptophan biosynthesis | 2.20E-05 |
| 10480 | Glutathione metabolism | 5.35E-05 |
| 10592 | alpha-Linolenic acid metabolism | 1.75E-08 |
| 10710 | Carbon fixation | 2.88E-03 |
| 10770 | Pantothenate and CoA biosynthesis | 1.58E-03 |
| 10950 | Alkaloid biosynthesis I | 2.68E-03 |
| 11000 | Single reactions | 1.48E-06 |
| 11002 | Auxin biosynthesis | 1.75E-03 |
| 50109 | transport systems | 9.27E-04 |
| 60066 | WRKY | 1.69E-05 |

**Profile F**

| VVID | Molecular network | p-value |
|---|---|---|
| 10500 | Starch and sucrose metabolism | 1.01E-04 |
| 23410 | Base excision repair | 4.50E-03 |
| 30003 | Auxin signaling | 6.03E-03 |

Figure 3.4. Profiles of grapevine berry trancripts at hard-green and ripe stages in reponse to *B. cinerea* inoculation. K-means clustering of grapevine genes based on the cosine distance of their log2 (fold change) values. Genes that showed at least twofold expression difference with p-value < 0.01 were considered, and clustered into 12 clusters. The clusters were grouped into 6 major profiles (A, B, C, D, E and F). Molecular enrichment analysis based on VitisNet is provided for each group.

### 3.4.3. Transcriptional alterations of *B. cinerea* during quiescent infection, at 4 wpi

The signal of *B. cinerea* transcriptome detected in the inoculated samples at 4 wpi was very low; the reason could largely be linked to a reduced fungal biological activity as well as to the little fungal mass present at the quiescent stage. Only about 20 % of *B. cinerea* genes (1,926) had at least 1 raw reads in all of the three biological replicates. Within this set, those represented by an average of at least 10 reads (289), considered as expressed fungal transcript at 4 wpi and functionally annotated using Blast2GO (Conesa et al., 2005) and Amselem et al. (2011) (Supplemental Table S3.6). Using the Combined Graph Function of Blast2GO, metabolic processes, structural constituent of ribosome, and intracellular GO slim terms were represented most in the 289 genes (Supplemental Table S.3.9). Twenty-two genes from this group, selected based on their function, are presented in Table 3.1; the expression profile of 9 of them, involved in functions such as cell wall metabolism, redox-reaction, and transcriptional regulation, was further examined using qPCR assay (Figure 3.5). As depicted in Figure 3.5, all but *Bcin07g01540* and *Bcin13g05810* had a higher relative expression during quiescent infection at hard green stage than during initial infection at flowering and pre-egression and egression stages at ripe.

Table 3.1. RNA-seq reads of selected *B. cinerea* transcripts with different biological functions at quiescence in the hard-green berry (4 wpi)

| | | RNA-seq reads (average) | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | 12 wpi | | |
| Gene ID | Function (Blast2GO) | 24 hpi | 96 hpi | 4 wpi | Ripe_Peg* | Ripe_ Eg | PDB culture |
| Bcin01g09570 | yt521-b-like splicing | 9 | 8 | 15 | 119 | 2,685 | 2,177 |
| Bcin02g06140 | CP2 transcription factor protein | 2 | 6 | 12 | 2 | 1,773 | 3,193 |
| Bcin02g06930 | 1,3-beta-glucan synthase | 49 | 27 | 20 | 24 | 15,886 | 12,324 |
| Bcin03g01920 | catalase | 19 | 6 | 32 | 12 | 7,577 | 1,314 |
| Bcin03g06170 | 40S ribosomal protein | 108 | 62 | 21 | 42 | 18,774 | 34,625 |
| Bcin03g07630 | 60S ribosomal protein | 97 | 48 | 21 | 19 | 14,335 | 26,262 |
| Bcin03g07670 | NAD-specific glutamate dehydrogenase | 30 | 20 | 31 | 10 | 2,937 | 11,142 |
| Bcin04g01310 | GPI-anchored cell wall organization Ecm33 | 97 | 34 | 45 | 37 | 31,061 | 17,374 |
| Bcin07g01540 | elongation factor 2 | 114 | 68 | 42 | 25 | 21,912 | 35,265 |
| Bcin07g03980 | chitin- domain 3 | 22 | 12 | 67 | 7 | 3,776 | 2943 |
| Bcin08g05540 | ASG1; CND1, similar to Gas1-like protein | 129 | 54 | 410 | 35 | 24,761 | 35,778 |
| Bcin09g00200 | glucan endo-1,3-beta-glucosidase eglC | 15 | 15 | 31 | 8 | 5,578 | 8,505 |
| Bcin10g01180 | ABC transporter ATP-binding ARB1 | 21 | 14 | 20 | 19 | 10,154 | 5,384 |
| Bcin11g04800 | chitin deacetylase | 10 | 3 | 67 | 4 | 2,385 | 210 |
| Bcin11g04930 | Stress response ish1 | 7 | 6 | 21 | 19 | 15,610 | 348 |
| Bcin12g06170 | allergen; Peptide transport PTR2 | 8 | 2 | 20 | 1 | 682 | 86 |
| Bcin13g05580 | alcohol dehydrogenase 1 | 100 | 47 | 21 | 4 | 4,198 | 37,002 |
| Bcin13g05810 | aldehyde dehydrogenase | 238 | 46 | 48 | 25 | 10,689 | 4,569 |
| Bcin14g04260 | cell surface; Gas2, regulated by Bmp1 MAP kinase cascade | 18 | 4 | 112 | 23 | 21,022 | 41 |
| Bcin14g03970 | 1,3-beta-glucanosyltransferase gel1; glycolipid-anchored surface protein 5 precursor | 76 | 42 | 26 | 14 | 14,303 | 11,200 |
| Bcin15g02080 | ATP synthase beta chain | 107 | 67 | 27 | 24 | 19,251 | 36,547 |
| Bcin16g02430 | carbohydrate-binding module family 5 | 13 | 14 | 28 | 5 | 4,352 | 7,622 |

* The sequence depth of the samples was double; only two biological replicates were considered.

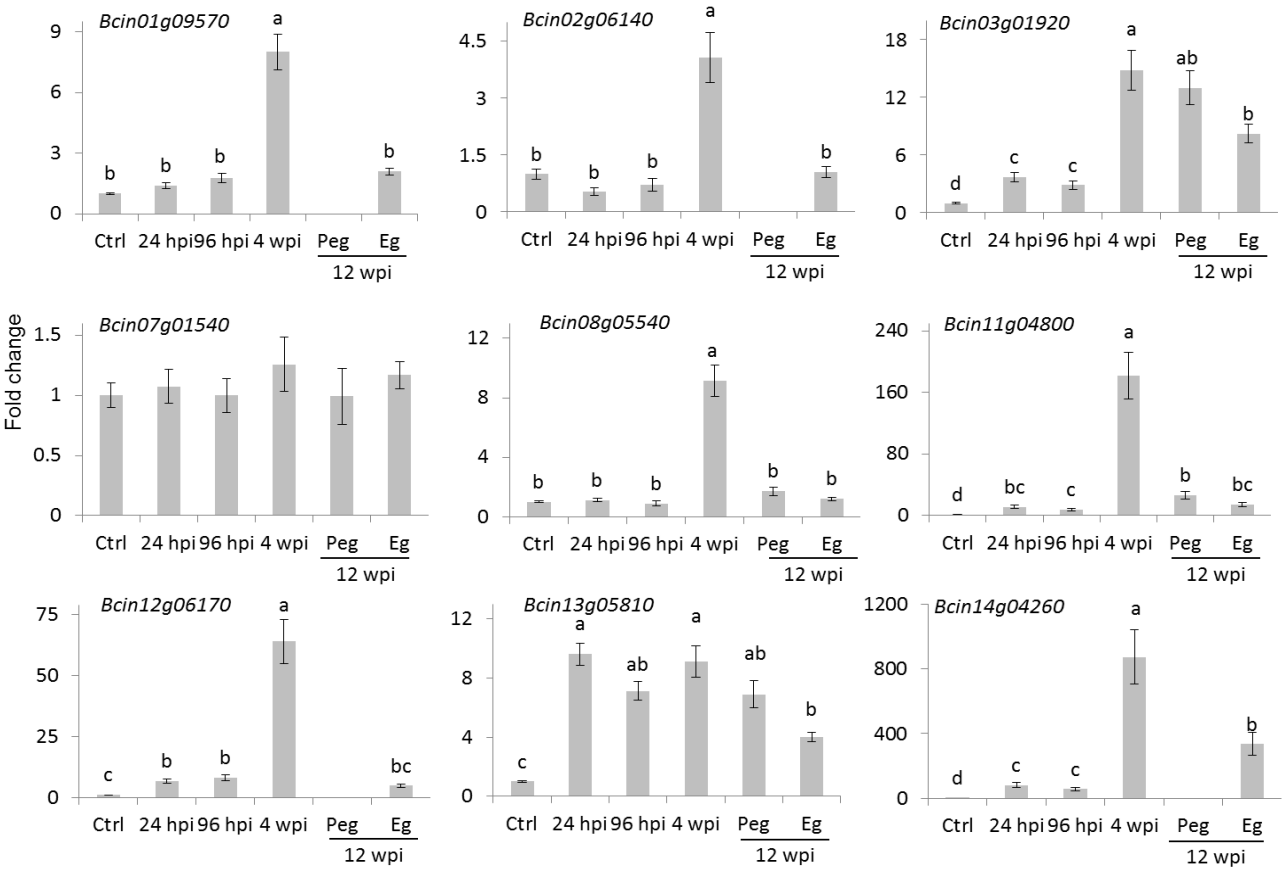



Figure 3.5. Expression profile of selected *Botrytis cinerea* genes having higher raw reads at hard-green berry stage relative to PDB-cultured *B. cinerea*. Bars represent fold change of samples at 24 and 96 hours post inoculation (hpi; flower infection), 4 weeks post inoculation (wpi; quiescent infection at hard-green berry stage), and 12 wpi (pre-egression, Peg, and egression, Eg, stages of *B. cinerea* at ripening) relative to the PDB-cultured *B. cinerea* (Ctrl). Normalization based on the expression levels of ribosomal protein L5, *BcRPL5*, and α tubulin, *BcTUBA*, was carried out before calculating fold changes. Error bar represents standard error of the mean of three biological replicates. Expression values followed by a common letter are significantly not different between samples, according to Tukey's Honestly Significant Difference test ($P \leq 0.05$), using one way ANOVA of log2(NRQ).

The qPCR results suggested that the biological activities of the fungus were not totally switched off during quiescent infection. The expression of *Bcin01g09570* and *Bcin07g01540* gene, which encode putative yt521-b-like splicing and elongation factor 2 proteins, respectively, and the numerous genes encoding for ribosomal proteins (Table 3.1 and Supplemental Table S3.6) indicated that protein synthesis activities were carried out during quiescent infection stage. The expression of stress and defense related genes, such as *Bcin12g06170*, encoding a protein similar to allergen, and *Bcin11g04800*, which encodes a putative chitin deacetylase protein, highlighted that the interaction between the pathogen and the plant was not passive. Chitin deacetylase

activity has been speculated to be involved in protecting the fungal cell wall from degradation by plant chitinases in other fungi (Deising and Siegrist, 1995; El Gueddari et al., 2002). In addition, genes encoding putative proteins involved in cell wall remodeling and integrity (for example, *Bcin04g01310* and *Bcin14g03970*) seemed also active during fungal quiescence (Table 3.1 and Supplemental Table S3.6). Stress usually causes ROS production that can lead to accumulation of aldehydes and alcohols in fungal cells (Asiimwe, et al., 2012). *Bcin13g05810* and *Bcin13g05580*, encoding putative aldehyde dehydrogenase and alcohol dehydrogenase, respectively, involved in the detoxification of alcohols and aldehydes, as well as *Bcin03g01920*, encoding a putative catalase, were speculated to help overcome stress from the berries' defense. Moreover, *Bcin08g05540*, encoding putative CND1 protein, *Bcin14g04260*, annotated for a putative cell surface protein and Gas2, and *Bcin02g06140*, encoding a putative CP2 transcription factor protein, all these genes probably involved in maintaining cell wall, (Garrett-Engele et al., 1995; Paré et al., 2012), appeared to be relatively expressed more during quiescent infection at 4 wpi as compared to initial and egression stages of infection (Figure 3.5).

The extremely low number of *B. cinerea* genes expressed *in planta* seconded by absence of any macroscopically noticeable disease progress at 4 wpi suggests that the pathogen was at its basal metabolic activity despite specific stress related genes were expressed.

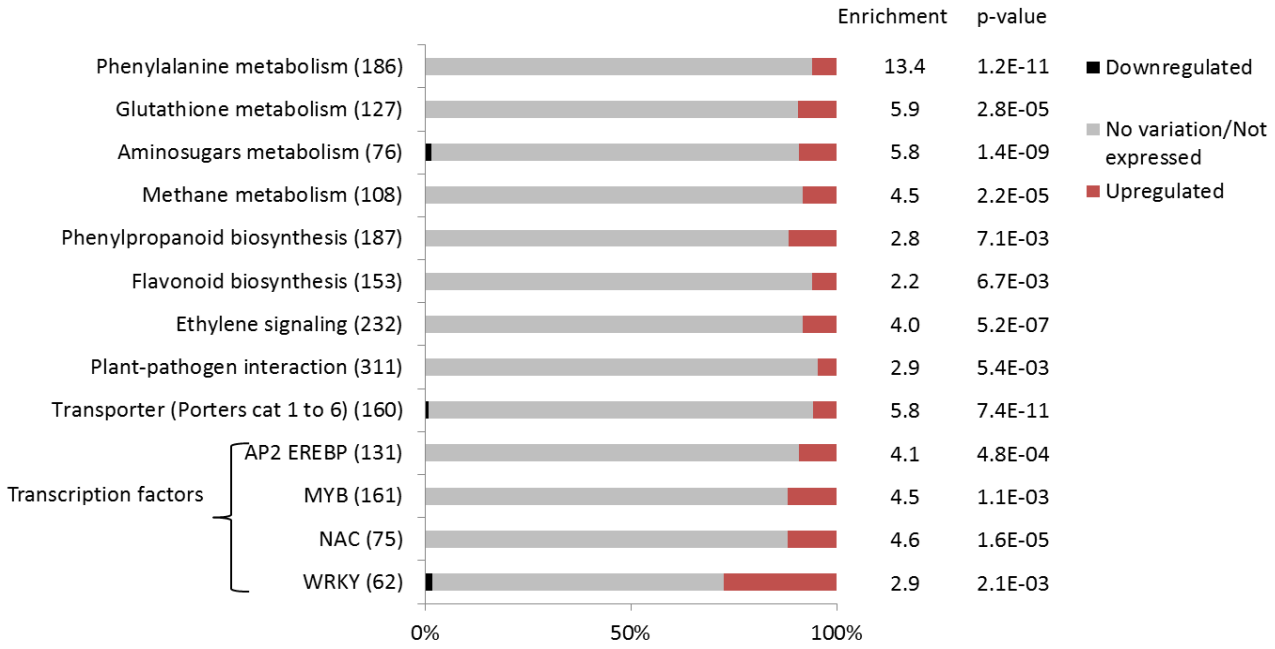



Figure 3.6. VitisNet functional classes enriched in the differentially expressed genes at hard-green berry stage due to quiescent *Botrytis cinerea*. The number presented in the parentheses is the total number of *V. vinifera* genes within each category. The portion of upregulated and downregulated genes within a category is represented by red and black bars, respectively

### 3.4.4. Response of hard-green berries to quiescent *B. cinerea*, at 4 wpi

In contrast to the fungus for which not many transcripts were observed, 599 grapevine genes were differentially regulated due to the quiescent presence of *B. cinerea*, of which only 21 genes were downregulated (Figure 3.3C and Supplemental Table S3.3). In this set of *Botrytis*-induced genes functional classes related to responses to stress, amino acid metabolism for redox activity and phenylpropanoid pathways, signaling, and TFs were overrepresented (Figure 3.6 and Supplemental Table S3.10). The visualization of individual gene responses in biotic stress pathway using MapMan tool also indicated a remarkable induction of genes related to signaling, TFs, proteolysis, PR-proteins and secondary metabolism (Figure 3.7). Genes involved in some defense pathways, which were activated at initial infection stage (24 hpi) but returned to basal level toward the entry of quiescence (96 hpi) during flower infection (Chapter 2), seemed to be reactivated at 4 wpi, such as genes in the phenylpropanoid biosynthesis pathway for stilbenoids, flavonoids and lignin precursors synthesis.

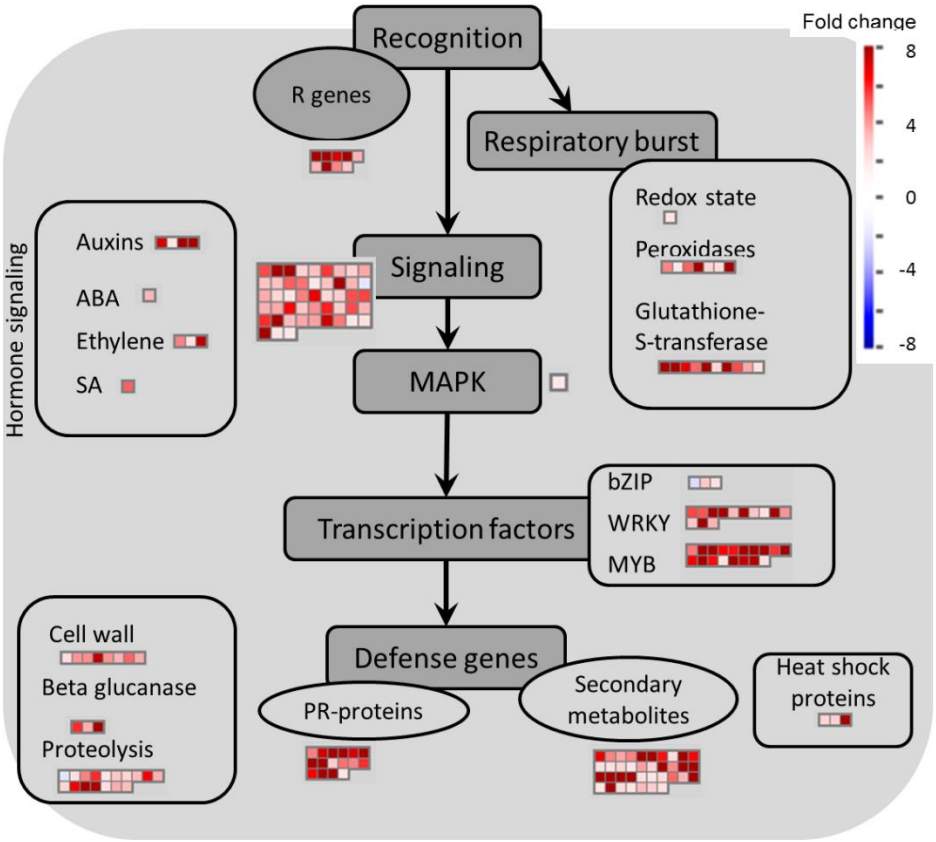



Figure 3.7. Biotic stress overview of hard-green berries due to quiescent *B. cinerea* infection, 4 wpi, as visualized by MapMan. Upregulated and downregulated genes are shown in red and blue, respectively. The scale bar displays fold change values. ABA, abscisic acid; MAPK, mitogen-activated protein kinase; SA, salicylic acid.

Table 3.2. Selected differentially expressed grapevine genes due to quiescent *B. cinerea* infection (at 4 wpi)

| ID | Fold change (log2) | Functional annotation |
|---|---|---|
| **Recognition and signaling** | | |
| VIT_05s0020g04680 | 1.07 | Auxin-induced protein 22D |
| VIT_12s0055g01280 | 1.98 | Brassinosteroid insensitive 1-associated receptor kinase 1 |
| VIT_14s0030g02150 | 4.22 | Calmodulin |
| VIT_03s0038g01380 | 1.02 | Calcium-binding EF hand |
| VIT_08s0056g00290 | 1.07 | Calcium-binding protein CML |
| VIT_04s0008g00440 | 1.78 | Clavata1 receptor kinase (CLV1) |
| VIT_11s0016g03080 | 2.51 | Clavata1 receptor kinase (CLV1) |
| VIT_06s0004g06210 | 5.07 | CYP86A1 |
| VIT_11s0118g00160 | 3.65 | Disease resistance protein |
| VIT_17s0000g07560 | 1.10 | EDS1 (Enhanced disease susceptibility 1) |
| VIT_08s0040g00920 | 3.67 | Glutathione S-transferase 25 GSTU7 |
| VIT_04s0044g01990 | 1.42 | Lectin protein kinase |
| VIT_18s0072g00990 | 1.30 | Leucine-rich repeat protein kinase |
| VIT_18s0122g01260 | 1.68 | Protein kinase 1B |
| VIT_10s0071g00150 | 1.60 | R protein disease resistance protein |
| VIT_00s0262g00010 | 2.25 | Receptor kinase RK20-1 |
| VIT_17s0000g02360 | 2.42 | Receptor protein kinase |
| VIT_16s0039g01310 | 2.18 | Receptor serine/threonine kinase PR5K |
| VIT_16s0148g00140 | 1.47 | Ser/Thr receptor-like kinase1 |
| VIT_17s0000g04400 | 3.10 | Wall-associated kinase 1 (WAK1) |
| VIT_17s0000g03340 | 1.08 | Wall-associated kinase 4 |
| **Trascription factors** | | |
| VIT_05s0049g00510 | 2.01 | Ethylene response factor ERF1 |
| VIT_05s0049g01020 | 1.55 | Myb domain protein 14 |
| VIT_15s0046g00170 | 1.21 | MYBPA1 protein |
| VIT_04s0008g02710 | 4.25 | NAC domain containing protein 39 |
| VIT_12s0028g00860 | 3.00 | NAC domain containing protein 42 |
| VIT_04s0008g05760 | 1.81 | WRKY DNA-binding protein 18 |
| VIT_08s0058g00690 | 1.61 | WRKY DNA-binding protein 33 |
| VIT_08s0058g01390 | 1.74 | WRKY DNA-binding protein 70 |
| **Transport** | | |
| VIT_08s0040g02840 | 4.46 | ABC transporter G member 29 |
| VIT_08s0058g00150 | 2.54 | Ammonium transporter 2 |
| VIT_04s0069g00540 | 1.90 | Glutamate receptor protein |
| VIT_01s0127g00070 | 1.17 | Nitrate transporter 2.5 |
| VIT_17s0000g09470 | 1.40 | Nitrate transporter 3.1 |
| VIT_03s0091g01050 | 1.53 | Nucleobase-ascorbate transporter 4 |
| **Cell wall** | | |
| VIT_05s0049g00010 | 1.23 | Cellulose synthase CSLG2 |
| VIT_00s0340g00050 | 2.60 | Endo-1,4-beta-glucanase korrigan (KOR) |
| VIT_14s0128g00690 | 4.20 | Germin protein 3 |
| VIT_06s0004g01990 | 3.06 | Proline-rich extensin-like family protein |
| VIT_12s0055g00200 | 2.20 | UDP-glucose glucosyltransferase |
| **Response to stress and secondary metabolism** | | |
| VIT_11s0052g01110 | 2.69 | 4-coumarate-CoA ligase 1 |
| VIT_16s0050g02220 | 3.18 | Acidic endochitinase (CHIB1) |
| VIT_03s0017g02110 | 3.16 | Anthocyanidin 3-O-glucosyltransferase |
| VIT_08s0007g06060 | 2.55 | Beta 1-3 glucanase |
| VIT_16s0098g00850 | 1.40 | Caffeic acid O-methyltransferase |
| VIT_03s0063g00140 | 1.19 | Caffeoyl-CoA O-methyltransferase |
| VIT_16s0100g00860 | 4.82 | Chalcone synthase |
| VIT_05s0094g00340 | 2.17 | Chitinase class IV |
| VIT_09s0070g00240 | 1.19 | Cinnamoyl-CoA reductase |
| VIT_06s0004g01010 | 3.95 | Dirigent protein pDIR9 |
| VIT_07s0031g01380 | 3.72 | ferulate 5-hydroxylase |
| VIT_16s0098g00860 | 2.31 | Flavanone 3-hydroxylase |
| VIT_02s0109g00310 | 1.84 | flavonoid 3-monooxygenase |
| VIT_10s0003g00470 | 2.15 | Isoflavone methyltransferase |
| VIT_13s0067g01970 | 2.78 | Laccase |
| VIT_17s0000g06290 | 4.78 | Lipase GDSL |
| VIT_02s0025g04250 | 4.12 | Osmotin |
| VIT_05s0077g01570 | 4.37 | Pathogenesis protein 10 [Vitis vinifera] |
| VIT_03s0088g00710 | 3.50 | Pathogenesis-related protein 1 precursor (PRP 1) |
| VIT_04s0023g02570 | 2.15 | Peroxidase 72 |
| VIT_16s0039g01100 | 4.90 | Phenylalanin ammonia-lyase [Vitis vinifera] |
| VIT_02s0025g00750 | 3.49 | Pinoresinol forming dirigent protein |
| VIT_00s0480g00030 | 2.75 | Polyphenol oxidase |
| VIT_16s0100g01200 | 5.33 | Stilbene synthase |
| VIT_02s0025g02850 | 1.16 | Subtilisin protease |
| VIT_02s0025g04270 | 4.33 | Thaumatin |

Receptor-like protein kinases (RLKs) regulate recognition and responses to both internal and external signals, and also take part in defense and symbiosis. From the differentially expressed genes, about one-sixth of them were those involved in signaling, dominated by RLKs (Table 3.2 and Supplemental Table S3.3). Membrane-localized RLKs which were switched on during infection initiation at flowering, like *Clavata1 receptor kinase (CLV1)*, *Brassinosteroid insensitive 1– associated kinase 1 (BAK1)*, and *Wall-associated kinase 1 (WAK1)*, were also upregulated during the interaction at quiescent infection. The involvement of *WAK1*, a damage associated pattern receptor which recognize plant cell wall–derived oligogalacturonides due to cell wall degradation (Brutus et al., 2010), at this stage of interaction was not obvious, however. From the transcriptional alteration on RLKs, lectin protein kinase and protein kinase 1, seem to be quiescent-stage-specific. The putative orthologue in other species are associated to plant immunity, in particular the latter is known to mediate signaling in response to *B. cinerea* (Abuqamar et al., 2008). In addition, transcripts related to $Ca^{2+}$ mediated signaling (such as calcium- and calmodulin-binding proteins) and oxidative stress (mainly GST and cytochrome P450 monooxygenases) were also found to involve in the ongoing berry immunity.

Key TFs that play an important role in plant-microbe interaction, which were also upregulated at initial infection during flowering, were still tuned on in the presence of asymptomatic *B. cinerea*, at 4 wpi (Table 3.2 and Supplemental Table S3.3). Most prominent was the MYB TF family: 21 genes encoding 14 different MYB proteins, including *MYB14* and *MYBPA1* which respectively regulate stilbene and proanthocyanidin biosynthesis. From the WRKY and NAC TFs *WRKY33*, which regulates plant defense reaction against pathogens (Birkenbihl et al., 2012; Merz et al., 2015), *NAC042*, known to regulate phytoalexin biosynthesis (Saga et al., 2012), *WRKY51*, mediating the repression of JA signaling in a SA- and low-oleic-acid-dependent manner (Gao et al., 2011), and *NAC036*, *WRKY18* and *WRKY70*, regulate SA biosynthesis and SA signal transduction (Wang et al., 2006) were involved. The upregulation of SA signaling marker genes, such as *PR1* and *EDS1*, is a further indication of SA involvement in enhancing the defense ability of the hard-green berry. In addition, the upregulation of 3 *ACC oxidase* and 12 *AP2/ERF* genes underlines that also ET signaling is in place during the interaction at quiescent state. AP2/ERF are vital in plant-pathogen interactions (Gutterson and Reuber, 2004; Licausi et al., 2013). Genes related to auxin metabolism were also modulated, suggesting that also this phytohormone is likely involved.

Intriguingly, differential regulation was observed for genes coding for various PR proteins and enzymes of the phenylpropanoid pathway and of flavonoid and stilbenoid biosynthesis during quiescent infection (Table 3.2 and Supplemental Table S3.3). For some of the genes, the upregulation was nearly 50 fold. PR proteins encoding genes included: PR 10s and the regulator WRKY33, involve in defense against *B. cinerea* and *Plasmopara viticola* in grapevine (Merz et al., 2015; ?firstpaper?); β -1,3-glucanase and different classes of chitinases, to attack fungal cell wall; and osmotin and thaumatin, known to interfere with the growth of *Phomopsis viticola* and *Botrytis cinerea* mycelia (Monteiro et al., 2003). Besides, a gene encoding cystatin, proteinaceous inhibitors of cystein proteases which is suggested to inhibit the growth of *B. cinerea* (Pernas et al., 1999), was also upregulated.

Table 3.3. Concentration (µg/g fresh weight) of selected polyphenolic secondary metabolites in mock (Ctrl) and *B. cinerea* (Trt) inoculated grapevine at hard-green berry stage, 4 wpi.

| | | | Hard green berry (4 wpi) | |
|---|---|---|---|---|
| Class | Secondary metabolites | LOD(µg/g) | Ctrl | Trt |
| stilbenoids | *trans*-resveratrol | 0.005 | n.d. | 1.52 ± 0.47 |
| | *trans*-piceide* | 0.0125 | 0.97 ± 0.09 | 10.08 ± 1.83 |
| | *cis*-piceide* | 0.0025 | 0.34 ± 0.05 | 1.7 ± 0.40 |
| | astringin* | 0.005 | 0.14 ± 0.03 | 1.55 ± 0.26 |
| | isorhapontin | 0.005 | n.d. | 0.21 ± 0.06 |
| | arbutin* | 0.005 | 0.13 ± 0.01 | 0.90 ± 0.19 |
| | *trans*-ε-viniferin | 0.005 | n.d. | 9.86 ± 2.16 |
| | *cis+trans*-o-viniferin | 0.0125 | n.d. | 0.30 ± 0.07 |
| | pallidol | 0.005 | n.d. | 0.60 ± 0.19 |
| | ampelopsin D+quadrangularin A | 0.025 | n.d. | 2.78 ± 0.81 |
| | α-viniferin | 0.0125 | n.d. | 1.86 ± 0.66 |
| | E-*cis*-miyabenol | 0.0125 | n.d. | 0.91 ± 0.34 |
| | Z-miyabenol C | 0.05 | n.d. | 0.40 ± 0.12 |
| | isohopeaphenol | 0.0125 | n.d. | 0.16 ± 0.05 |
| Flavonoids | naringenin* | 0.0025 | 0.01 ± 0.00 | 0.08 ± 0.01 |
| | catechin | 0.025 | 17.75 ± 1.23 | 26.06 ± 3.55 |
| | epicatechin* | 0.025 | 4.40 ± 0.44 | 9.33 ± 1.19 |
| | epicatechin gallate* | 0.025 | 0.10 ± 0.02 | 1.67 ± 0.29 |
| | procyanidin B1 | 0.0125 | 67.98 ± 4.47 | 136.63 ± 28.81 |
| | taxifolin | 0.0025 | 6.54 ± 0.95 | 13.40 ± 3.46 |

LOD, limit of detection; wpi, week post inoculation; values after ± are standard error.
Asterisks (*) indicate statistically significant difference ($P < 0.05$)
n.d., not determined. For metabolites that were not determined in control sample, no statistical comparison was made.

In the case of secondary metabolites biosynthesis, though there was generally a smaller number of DE genes involved as compared to the initial infection during flowering, still the monolignol, flavonoid and stilbenoid biosynthesis pathways were somehow active. Genes encoding enzymes in the biosynthetic pathway, for example, phenylalanine ammonia-lyase, stilbene synthase, anthocyanidin 3-O-glucosyltransferase, chalcone synthase, and cinnamoyl-CoA reductase were differentially regulated (Supplemental Table S3.3). Many compounds of the phenylpropanoid pathway were also quantified by UHPLC-DAD-MS (Supplemental Table S3.11). The strongest effect was recorded on flavonoids and stilbenoids (Table 3.3), in particular on compounds known to mediate defense against pathogens: resveratrol, viniferin, ampelopsin, miyabenol, isohopeaphenol, catechin, and proanthocyanidins (Jersch et al., 1989; Goetz et al., 1999; Pezet et al., 2003a; Favaron et al., 2007; Hammerbacher et al., 2011). For the stilbenoid class, most of the compounds were below the detection limit in control samples. Here it should be noted that there is ontogenic reduction of the polyphenols' concentration between flower and hard-green developmental stages (Supplemental Table S2.8 and S3.11).

In sum, even though we did not observe any known virulence related genes of the pathogen which can provoke response from the berries at 4 wpi, it seemed, however, that the berries are at primed state with enhanced immunity as it recognizes nonself organism, which in turn help the berry to contain the pathogen. This gives an interesting insight that there is a molecular communication going on between the quiescent *B. cinerea* and the berry.

### 3.4.5. *B. cinerea* transcriptome during pre-egression and egression stages, at ripening

At ripening, two kinds of berries were collected from *Botryits*-treated samples: berries without visible *B. cinerea* outgrowth (samples with pre-egressed *B. cinerea*) and berries with visible signs of *B. cinerea* outgrowth (named egressed *B. cinerea*). Both samples were subjected to RNA-seq to analyze the dual transcriptome (fungus and plant) at these stages. In the samples with pre-egressed *B. cinerea*, as in flower and hard-green berry samples, the number of fungal transcripts was unfortunately very low, even though the sequencing depth was doubled. Moreover, in one of the biological replicates (replicate 2) the growth of the fungus was advanced than the rest two, as inferred from the number of fungal transcripts obtained from the RNA-seq experiment (Supplemental Table S3.2 and S3.7). Taking similar threshold previously used in the flower and hard-green berry samples (an average of at least 10 reads) in the two biological replicates,

excluding the sample with advanced *B. cinerea* growth, 431 genes were selected for further analysis. The functional annotation and the most represented functional classes in these set of genes are provided in Supplemental Table S3.7 and S3.12, respectively.

Within the 431 genes, there were several genes encoding proteins functionally associated to the infection process: dyp-type peroxidase and galactose oxidase, involved in generation and detoxification of ROS (Schumacher et al., 2015); polygalacturonase, deployed in pectin degradation; glyoxal oxidase and oxalate decarboxylase, both catalyze oxalate; different types of oxidoreductases; and cerato-platanin *BcSPL1*, a small protein required for full virulence (Frías et al., 2011, 2014) . Interestingly, these genes showed a low number of raw reads or were not expressed at all at 4 wpi (Table 3.4), indicating that the physiological state of the fungus in the ripe berry before egression was different from that in the hard-green one.

Table 3.4. RNA-seq reads of selected *B. cinerea* transcripts having more reads at pre-egression (12 wpi, on ripe berry) than at quiescence (4 wpi, on hard-green berry).

| | | | RNA-seq reads (average) | | |
|---|---|---|---|---|---|
| | | | 4 wpi | 12 wpi (Peg)* | PDB culture |
| Gene ID | Function (Blast2GO) | Further description | | | |
| Bcin13g05720 | Dyp-type peroxidase | BcPRD1, Dyp-type peroxidase | 2 | 42 | 312 |
| Bcin13g05710 | Galactose oxidase beta-propeller | BcGOX1, Galactose oxidase | 4 | 30 | 609 |
| Bcin12g02040 | acid protease | BcAP8, aspartic proteinase | 1 | 12 | 1,064 |
| Bcin14g00850 | polygalacturonase | Pectin degradation | 0 | 30 | 147,821 |
| Bcin07g01150 | ubiquitin-60S ribosomal L40 | Polyubiquitin | 11 | 23 | 30,510 |
| Bcin06g01930 | glyoxal oxidase | Glyoxal oxidase | 3 | 31 | 6,239 |
| Bcin15g02380 | acid protease partial | Glutamic protease | 0 | 42 | 56 |
| Bcin01g09650 | glucose-6-phosphate 1-dehydrogenase | Glucose-6-phosphate dehydrogenase | 15 | 36 | 6,740 |
| Bcin03g00500 | probable rot1 PRECURSOR | Cerato-platanin family protein BcSpl1 | 5 | 29 | 64,662 |
| Bcin01g11220 | glycoside hydrolase family 17 | b-1,3-Glucosidase | 4 | 16 | 4,617 |
| Bcin07g00160 | glycoside hydrolase family 18 | CAZyme | 5 | 42 | 2,386 |
| Bcin04g05650 | oxalate decarboxylase family bicupin | | 1 | 41 | 38 |
| Bcin11g02720 | aldo keto reductase | Oxidoreductase | 9 | 35 | 3,377 |
| Bcin04g05700 | nadp-dependent alcohol dehydrogenase | Oxidoreductase | 1 | 21 | 4,844 |
| Bcin06g04940 | short-chain dehydrogenase | Oxidoreductase | 2 | 34 | 338 |
| Bcin09g03090 | lipid phosphate phosphatase 1 | Oxidoreductase | 2 | 25 | 152 |
| Bcin16g04720 | C-5 sterol desaturase | | 5 | 23 | 14,517 |
| Bcin11g02630 | phytanoyl- dioxygenase | Oxidoreductase | 0 | 23 | 22 |
| Bcin15g03620 | glycosyltransferase family 35 | CAZyme | 8 | 20 | 12,308 |
| Bcin11g06080 | ATP synthase H mitochondrial precursor | | 3 | 40 | 6,851 |

* The sequence depth of the samples was double; only two biological replicates were considered.

In the egressed sample, 86 % of total *B. cinerea* transcriptome was expressed, which is a sign of utmost metabolic activity. Interestingly, the number of genes expressed were similar between egressed and PDB cultured *B. cinerea*, about 10,000 of the total 11701 predicted genes (van Kan et al., 2016). Such en masse transcriptional activity was never seen in the other evaluated infection stages, possibly due to the low measurable signal linked to the little amount of the fungus, but likely also to a reduced transcriptional activity at these stages. In other words, at ripening, the time, the status of the host tissue, and the environmental conditions, components of the disease pyramid, were conducive for *B. cinerea* to egress and grow vigorously, as observed from Figure 3.1C.

Table 3.5. RNA-seq reads of selected virulence *B. cinerea* genes which were not differentially expressed in comparison to PDB culture d *B. cinerea*

| | | RNA-seq reads (average) | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | 12 wpi | | |
| Gene ID | Gene name | 24hpi | 96hpi | 4 wpi | Peg* | Eg | PDP culture |
| Bcin14g00850 | Polygalacturonase1 (BcPG1) | 209 | 175 | 0 | 30 | 24,289 | 147,821 |
| Bcin10g00740 | Glutathione S-transferase (BcGST1) | 9 | 1 | 3 | 4 | 1,496 | 2,655 |
| Bcin01g00060 | Botcinic acid6 (BcBOA6) | 13 | 2 | 0 | 1 | 740 | 1,048 |
| Bcin03g00500 | Cerato-platanin family protein (BcSPL1) | 138 | 75 | 5 | 29 | 29,201 | 64,662 |

* The sequence depth of the samples was double; only two biological replicates were considered.

Compared to the transcriptional changes of *B. cinerea* cultured in PDB, 3,548 genes were differentially regulated during egression at ripening (Figure 3.3C and Supplemental Table S3.5). These DE genes are overrepresented in metabolic processes, ion binding, catalytic and oxidoreductase activities, cytoplasm, intracellular part functional classes (Supplemental Table S3.13). Genes encoding carbohydrate-active enzymes and others involved in plant cell wall degradation (Espino et al., 2010; Blanco-Ulate et al., 2014), such as *Bcin10g06130* and *Bcin14g01630*, encoding pectinases, *Bcin03g01680*, encoding a polygalacturonase, and *Bcin07g06480* and *Bcin15g03080*, encoding cutinases, were expressed more during egression than in PDB medium. Other virulence and/or growth related genes having similar expression trend as those mentioned above were: ROS producers and scavengers like *Bcin03g03390*, *Bcin13g05710*, and *Bcin13g05720* (Rolke et al., 2004; Schumacher et al., 2015); characterized aspartic proteases (*Bcin12g02040* and *Bcin12g00180*, ten Have et al., 2010); membrane

transporters, mostly the ATP-binding cassette; and botcinic acid and botrydial phytotoxins (*Bcin12g06390* and *Bcin12g06380*, Siewers et al., 2005; Dalmais et al., 2011). On the other hand, known virulence genes like *BcPG1* (*Bcin14g00850*), *BcGST1* (*Bcin10g00740*), *BcBOA6* (*Bcin01g00060*), and *BcSPL1* (*Bcin03g00500*) had similar or lower expression level during egression as compared to PDB cultured *Botrytis*. This, however, does not mean that they do not play a role in the necrotrophic stage of infection during egression at ripening, as the number of reads of these genes was reasonably high at this stage (Table 3.5).

### 3.4.6. Response of ripe berry for *B. cinerea*'s pre-egression and egression state

Grapevine berries responded to the necrotrophic colonization of the fungus, during egression, by reprogramming the transcription of 2,213 genes (Figure 3.3C and Supplemental Table S3.4). Of these genes, 1,564 were already differentially regulated in pre-egressed samples, together with additional 83 genes. The GO enrichment analysis computed on these DE genes of pre-egressed and egressed samples showed a high overlap in the enriched biological process: secondary metabolic process, response to stimulus, catabolic process and transport functional classes were among the enriched functional classes (Supplemental Table S3.14). In the case of the genes modulated between egression and pre-egression states (Eg. Vs. Peg), only 15 genes were not part of the DE genes obtained in the Peg Vs. Ctrl and Eg. Vs Ctrl comparisions (Figure 3.3C).

The differentially expressed genes, 2,311 in total at 12 wpi, were clustered into 8 groups based on their expression pattern considering control, pre-egressed, and egressed samples as shown in Supplemental Figure S3.2. Several functional classes as amino acid metabolism, protein processing, carbohydrate and lipid metabolism, phytohormone (auxin, ET, and JA) signaling were overrepresented, combinding the 8 clusters into into four main expression profiles (A-D in Supplemental Figure S3.2).

Almost all of the DE genes in the pre-egressed samples were also in the egressed samples, and there existed a general similar expression direction (up- or down-regulation) in these genes Supplemental Table S3.4 and Supplemental Figure S3.2). As a result, we used the the DE genes of the egressed stage only to visualize the biotic stress pathway (Figure 3.8), *via* MapMan, as it can give enough insight of the pre-egressed stage as well. Figure 3.8 depicted the transcriptional changes involved in biotic stress were huge. Although it might seem surprising, it is not uncommon to see such modulation of defense related genes in infected plant tissues even when

the pathogen wins the battle (Alkan et al., 2014; Agudelo-Romero et al., 2015; Kelloniemi et al., 2015). This could be due to the futile attempts of the infected tissue reacting against the pathogen and/or to the transcriptome attributes of other cell layers, not yet colonized, as it is impossible to spatially resolute. From the biotic stress pathway visualization, however, the presence of many downregulated genes in the PR protein and secondary metabolite categories, and the involvement of a lot of genes in JA and ET signaling and cell wall modification (Figure 3.8) were unique of the ripe berry transcriptome as compared to flower and hard-green berry due to *B. cinerea* (Figure 2.4 and Figure 3.7).

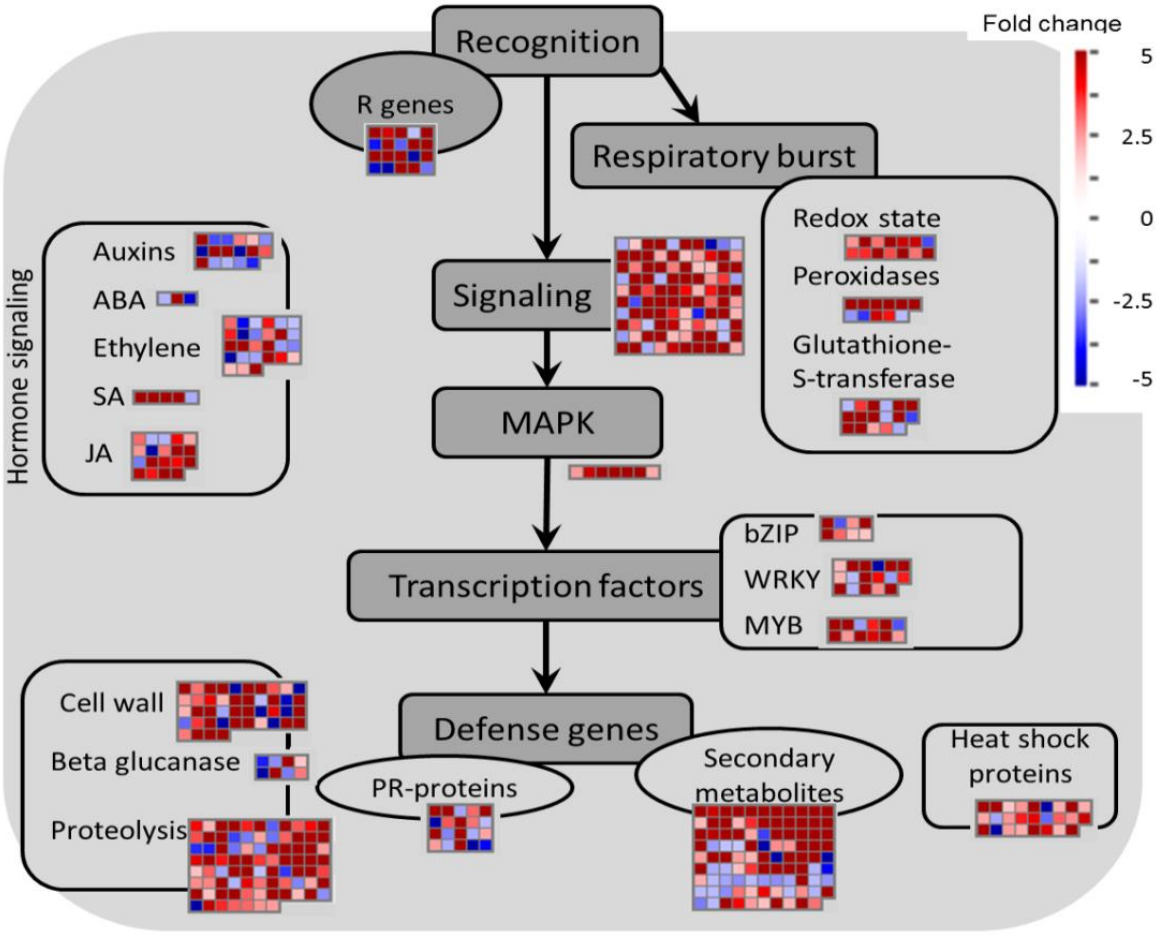



Figure 3.8. Biotic stress overview of ripe berries due to egressed *B. cinerea*, 12 wpi, as visualized by MapMan. Upregulated and downregulated genes are shown in red and blue, respectively. The scale bar displays fold change value. ABA, abscisic acid; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; SA, salicylic acid.

The PR proteins, which were differentially expressed by the infection during flowering and hard-green berry stages, were also regulated in ripe berry when during *Botrytis* egression, but to a different extent or direction and/or with reduced number of gene copies (Table 3.6). For example, two *PR10* genes, namely VIT_05s0077g01580 (upregulated) and VIT_05s0077g01690 (downregulated) were among the PRs in ripe berry; however, during initial infection at flowering, 5 colocalized PR10 genes, including VIT_05s0077g01690, were highly upregulated (up to 25 fold). Similar trend was observed for other PR genes as osmotin, thaumatin, and chitinases (Supplemental Table S2.3, S3.3 and S3.4). In addition, endochitinase and chitinase class I, which were upregulated during initial (flower) and quiescent (hard-green berry) infections, were found down regulated during *B. cinerea* egression.

The results also suggested that during *B. cinerea* egression the SA pathway was repressed, but the JA pathway was actively functioning. The expression of genes encoding PR1, an SA marker, and EDS1 , a defense regulator involving SA signaling (Wiermer et al., 2005), were downregulated or not affected. On the other hand, genes encoding jasmonate ZIM-domain1, a JA marker, and MYC2, involved in the activation of JA signaling (Lorenzo et al., 2004), were upregulated (Supplemental Table S3.4).

Table 3.6. Selected differentially expressed grapevine genes during pre-egression and egression of *B. cinerea* on ripe berries (12 wpi).

| ID | Fold change (log 2) Peg | Eg | Functional annotation |
|---|---|---|---|
| Transcription factors | | | |
| VIT_07s0141g00690 | 3.3 | 6.5 | ERF/AP2 transcription factor sub B-1 |
| VIT_10s0003g00580 | 2.9 | 2.8 | ERF/AP2 transcription factor sub B-3 |
| VIT_02s0234g00130 | | -1.1 | Ethylene responsive element binding factor 1 |
| VIT_07s0005g03340 | 2.8 | 2.9 | Myb domain protein 14 |
| VIT_19s0027g00870 | -1.3 | -0.9 | NAC domain-containing protein 42 |
| VIT_06s0004g07500 | 2.4 | 2.1 | WRKY DNA-binding protein 33 |
| Cell wall | | | |
| VIT_09s0002g01320 | -1.0 | -1.2 | Germin-like protein |
| VIT_14s0060g02750 | -2.0 | -3.3 | Germin-like protein 3 |
| VIT_14s0128g01020 | -0.8 | -1.5 | Germin-like protein 3 |
| VIT_06s0009g02570 | 0.9 | 2.9 | Pectinesterase family |
| VIT_02s0154g00600 | 1.1 | 3.1 | Pectinesterase family |
| VIT_04s0044g01020 | 4.5 | 11.6 | Pectinesterase family |
| VIT_07s0005g00720 | 4.7 | 8.5 | Pectinesterase family |
| VIT_08s0007g07770 | 4.8 | 7.2 | Polygalacturonase PG1 |
| VIT_08s0007g07760 | 5.0 | 8.3 | Polygalacturonase PG1 |
| VIT_11s0052g01180 | 0.7 | 3.2 | Xyloglucan endotransglucosylase |
| VIT_05s0062g00240 | 1.1 | 2.1 | Xyloglucan endotransglucosylase |
| Response to stress and secondary metabolism | | | |
| VIT_04s0023g03550 | 1.9 | 4.2 | Thaumatin |
| VIT_07s0005g02560 | | -1.1 | Chitinase Class I |
| VIT_05s0094g00360 | -0.8 | -1.5 | Chitinase class IV |
| VIT_05s0094g00200 | | 3.4 | Chitinase class IV |
| VIT_03s0038g03400 | | -1.2 | Endochitinase 1, basic |
| VIT_05s0077g01690 | | -1.1 | Pathogenesis protein 10 |
| VIT_05s0077g01580 | | 1.6 | Pathogenesis protein 10 |
| VIT_03s0088g00700 | | -1.2 | Pathogenesis related protein 1 precursor |
| VIT_03s0088g00690 | -3.4 | -3.5 | Pathogenesis-related protein 1 precursor |
| VIT_05s0077g01150 | | 1.5 | Beta-1,3-glucanase |

| ID | Fold change (log 2) Peg | Eg | Functional annotation |
|---|---|---|---|
| Response to stress and secondary metabolism | | | |
| VIT_08s0007g06040 | 4.6 | 6.7 | Beta-1,3-glucanase |
| VIT_18s0001g14790 | -4.0 | -4.4 | Lipase 3 (EXL3) family II extracellular |
| VIT_17s0000g10060 | -1.5 | -2.3 | Lipase GDSL |
| VIT_01s0137g00700 | | 1.5 | Lipase GDSL |
| VIT_14s0128g00080 | 4.5 | 5.6 | Lipase GDSL |
| VIT_16s0039g01320 | 4.98 | 5.07 | Phenylalanin ammonia-lyase |
| VIT_08s0040g01710 | 2.34 | 1.53 | Phenylalanine ammonia-lyase |
| VIT_00s2849g00010 | 5.84 | 4.39 | Phenylalanine ammonia-lyase |
| VIT_02s0025g03660 | -1.5 | -0.9 | 4-coumarate-CoA ligase |
| VIT_16s0050g00390 | 3.1 | 4.2 | 4-coumarate-CoA ligase |
| VIT_03s0063g01690 | -1.2 | -1.8 | flavonoid 3-monooxygenase |
| VIT_18s0001g14310 | 0.8 | 1.0 | Flavonone- 3-hydroxylase |
| VIT_04s0023g01290 | -0.9 | -1.5 | Anthocyanidin 3-O-glucosyltransferase |
| VIT_03s0017g02000 | 3.7 | 6.7 | Anthocyanidin 3-O-glucosyltransferase |
| VIT_03s0038g01460 | 0.7 | 1.2 | Chalcone synthase |
| VIT_02s0012g01570 | -1.5 | -0.7 | Cinnamoyl-CoA reductase |
| VIT_14s0066g01150 | 0.5 | 4.6 | Cinnamoyl-CoA reductase |
| VIT_00s0218g00010 | -0.4 | -1.2 | Cinnamyl alcohol dehydrogenase |
| VIT_13s0067g00680 | 0.3 | 2.1 | Cinnamyl alcohol dehydrogenase |
| VIT_06s0080g01000 | -2.0 | -1.6 | Secoisolariciresinol dehydrogenase |
| VIT_08s0058g00790 | 1.0 | 1.8 | Secoisolariciresinol dehydrogenase |
| VIT_01s0010g01960 | 8.01 | 3.82 | Anionic peroxidase |
| VIT_12s0055g00810 | -1.83 | -1.50 | Cationic peroxidase |
| VIT_08s0058g00970 | 1.98 | 0.96 | Cationic peroxidase |
| VIT_16s0100g00830 | | 1.1 | Stilbene synthase |
| VIT_16s0100g01200 | 7.1 | 10.5 | Stilbene synthase |
| VIT_16s0100g01190 | -1.0 | -2.0 | Stilbene synthase |
| VIT_16s0100g01160 | -0.8 | -1.2 | Stilbene synthase |
| VIT_16s0100g01140 | -0.8 | -1.4 | Stilbene synthase 2 |
| VIT_16s0100g00990 | 1.0 | 2.1 | Stilbene synthase 2 |

The secondary metabolites biosynthesis pathway, polyphenols in specific, was also generally affected by the egression of *B. cinerea* at ripening (Table 3.6 and Supplemental Table S3.4), although the transcription of genes encoding dihydroflavonol-4-reductase and flavanone 3-hydroxylase, both involved in flavonoid biosynthesis, was not affected. In line with this evidence, almost all of the quantified flavonoids were significantly lower or not different in the berries with egressed *B. cinerea* as compared to the control (Table 3.7). Whereas, for other key enzymes in the biosynthesis pathway mixed regulation (both up- and down regulation of genes encoding a given transcript) was observed, as in cinnamate 4-hydroxylase, 4-coumarate-CoA ligase 1, and stilbene synthase genes. For stilbene synthase, for example, from the 23 differentially-regulated stilbene synthase genes, 16 of them were downregulated. Overall, the trend of stilbenoids content of the samples was: for monomeric stilbenes (like trans-resveratrol, trans-piceide, and astringin), no appreciated difference between berries with egressed *Botrytis* and control; and for oligomeric stilbenes (like viniferin, E-cis-miyabenol, and isohopeaphenol), the concentration was highest in berries with pre-egressed *Botrytis*, followed by berries with egressed *Botrytis* and the lowest in healthy berries, control (Table 3.7).

Most of the genes involved in monolignol biosynthesis (such as CCR and CAD) were differentially regulated (Table 3.6 and Supplemental Table S3.4), though not all in the same direction—the mixed regulation mentioned above. However, a few but important genes like CCoAMT and F5H encoding genes were not differentially regulated. CCoAMT is involved in ferulic esterification and lignification process in response to pathogen attack in grapevine (Busam et al., 1997). From the secondary metabolite analysis, caffeate and ferulate, substrate for CCoAMT were not detected in any samples at ripening (Table 3.7). Perhaps, lignification is not an option in plant defense during ripening. The downregulation of all the differentially expressed genes encoding GLP3, an important protein in the oxidative cross-linking to strengthen cell wall during pathogen threat (Bradley et al., 1992; Godfrey et al., 2007), adds to the suggestion that cell wall apposition was downplayed at ripening. Rather, a number genes encoding proteins which involve in ripening-associated cell wall extensibility and disassembly like xyloglucan endotransglucosylase and polygalacturonase and pectinesterases (Nunan et al., 2001; Deytieux-Belleau et al., 2008) were highly upregulated in ripe berries with pre-egressed and egressed *Botrytis*. Pectinesterases is actually suggested to be involved both in cell wall loosening and strengthening (Micheli, 2001); however it seemed here it was involved in cell wall loosening as

there were a lot of upregulated polygalacturonases which degrade polygalacturonans, made accessible by the pectinesterases.

Table 3.7. Concentration (µg/g fresh weight) of polyphenolic secondary metabolites in mock (Ctrl) and berries with pre-egressed (peg) and egressed (eg) *B. cinerea* at ripe, 12 wpi.

| | | | Ripe berry (12 wpi) | | |
|---|---|---|---|---|---|
| Class | Compounds | LOD(µg/g) | Ctrl | Peg | Eg |
| benzoic acids | p-hydroxybenzoic acid | 0.005 | 0.61 ± 0.27 | 0.33 ± 0.04 | 1.2 ± 0.36 |
| | vanillic acid | 0.0025 | 0.00 ± 0.00 $^{b}$ | 0.16 ± 0.02 $^{b}$ | 0.72 ± 0.14 $^{a}$ |
| | gallic acid | 0.025 | 0.43 ± 0.01 | 0.18 ± 0.03 | 0.44 ± 0.16 |
| | 2,6-diOH-benzoic acid | 0.0025 | 0.22 ± 0.15 | 0.05 ± 0.02 | 0.04 ± 0.02 |
| | methyl gallate | 0.01 | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.02 ± 0.01 |
| Phenylpropanoids | caftaric acid | 0.0125 | 848.1 ± 84.86 $^{a}$ | 473.41 ± 16.29 $^{b}$ | 208.69 ± 60.81 $^{c}$ |
| | fertaric acid | 0.0025 | 16.63 ± 2.35 $^{a}$ | 15.47 ± 0.62 $^{a}$ | 6.14 ± 2.87 $^{b}$ |
| | trans-coutaric acid | 0.025 | 256.85 ± 8.89 $^{a}$ | 162.12 ± 10.76 $^{b}$ | 67.21 ± 20.07 $^{c}$ |
| Dihydrochalcones | phlorizin | 0.0025 | 1.82 ± 0.11 $^{a}$ | 1.6 ± 0.08 $^{a}$ | 0.7 ± 0.16 $^{b}$ |
| flavonoids | luteolin-7-O-Glc | 0.0025 | 0.24 ± 0.02 | 0.15 ± 0.03 | 0.07 ± 0.05 |
| | naringenin | 0.0025 | 0.24 ± 0.05 $^{a}$ | 0.04 ± 0.01 $^{b}$ | 0.03 ± 0.01 $^{b}$ |
| | catechin | 0.025 | 18.89 ± 6.61 | 6.29 ± 2.00 | 34.43 ± 19.75 |
| | epicatechin | 0.025 | 11.19 ± 1.33 | 7.86 ± 0.89 | 8.57 ± 2.9 |
| | epigallocatechin | 1.25 | 14.35 ± 0.96 $^{a}$ | 6.14 ± 0.61 $^{b}$ | 2.64 ± 0.63 $^{c}$ |
| | gallocatechin | 0.25 | 51.21 ± 5.8 $^{a}$ | 6.6 ± 0.83 $^{b}$ | 3.89 ± 2.14 $^{b}$ |
| | epigallocatechin gallate | 0.25 | 1.68 ± 0.04 | 1.3 ± 0.25 | 2.28 ± 1.35 |
| | epicatechin gallate | 0.025 | 1.33 ± 0.14 | 1.54 ± 0.28 | 12.12 ± 9.35 |
| | procyanidin B1 | 0.0125 | 81.82 ± 7.33 | 36.1 ± 8.23 | 44.26 ± 35.02 |
| | procyanidin B2 + B4 | 0.125 | 4.81 ± 0.45 | 1.47 ± 0.17 | 8.21 ± 3.3 |
| | procyanidin B3 (as B1) | 0.0125 | 28.78 ± 11 | 7.56 ± 0.84 | 24.58 ± 19.56 |
| | quercetin | 0.05 | 0.52 ± 0.04 $^{a}$ | 0.32 ± 0.07 $^{ab}$ | 0.17 ± 0.04 $^{b}$ |
| | taxifolin | 0.0025 | 98.19 ± 12.81 $^{a}$ | 40.59 ± 0.79 $^{b}$ | 19.54 ± 12.49 $^{b}$ |
| | myricetin | 1.25 | 8.66 ± 1.89 $^{a}$ | 4.3 ± 0.94 $^{ab}$ | 2.34 ± 0.39 $^{b}$ |
| | quercetin-3-Rha | 0.005 | 0.13 ± 0.03 | 0.23 ± 0.03 | 0.13 ± 0.07 |
| | quercetin-3-Glc+ quercetin-3-Gal | 0.0025 | 14.14 ± 1.77 | 14.97 ± 4.87 | 4.31 ± 1.59 |
| | isorhamnetin-3-Glc | 0.0025 | 2.22 ± 0.26 $^{b}$ | 10.65 ± 2.46 $^{a}$ | 2.79 ± 0.95 $^{b}$ |
| | rutin | 0.0025 | 2.06 ± 0.31 $^{a}$ | 1.28 ± 0.14 $^{ab}$ | 0.55 ± 0.26 $^{b}$ |
| | isorhamnetin-3-rutinoside | 0.005 | 0.27 ± 0.01 | 0.48 ± 0.13 | 0.14 ± 0.03 |
| | quercetin-3-glucuronide | 0.0125 | 31.2 ± 0.53 $^{a}$ | 7.51 ± 0.59 $^{b}$ | 2.17 ± 0.78 $^{c}$ |
| | isorhamnetin | 0.4 | 0.01 ± 0.00 | 0.01 ± 0.01 | 0.02 ± 0 |
| | syringetin | 0.025 | 0.06 ± 0.02 | 0.03 ± 0.01 | 0.01 ± 0 |
| | myricitrin | 0.5 | 1.13 ± 0.18 $^{a}$ | 1.03 ± 0.25 $^{a}$ | 0.31 ± 0.13 $^{b}$ |
| | syringetin-3-Glc+ syringetin-3-Gal | 0.05 | 6.01 ± 0.66 $^{b}$ | 17.51 ± 2.15 $^{a}$ | 5.12 ± 1.43 $^{b}$ |
| stilbenoids | arbutin | 0.005 | 4.96 ± 1.48 | 5.93 ± 1.52 | 3.74 ± 2.05 |
| | trans-resveratrol | 0.005 | 40.5 ± 9.34 | 36.94 ± 10.34 | 47.56 ± 23.86 |
| | cis-resveratrol | 0.0025 | 0.42 ± 0.07 $^{a}$ | 0.13 ± 0.06 $^{b}$ | 0.05 ± 0.02 $^{b}$ |
| | piceatannol | 0.0025 | 17.82 ± 6.04 | 22.24 ± 7.41 | 5.67 ± 2.95 |
| | trans-piceide | 0.0125 | 24.01 ± 2.01 $^{b}$ | 50.77 ± 3.69 $^{a}$ | 21.58 ± 9.08 $^{b}$ |
| | cis-piceide | 0.0025 | 13.04 ± 5.59 $^{a}$ | 11.59 ± 3.24 $^{a}$ | 2.97 ± 1.05 $^{b}$ |

Table 3.7. Continued.

| | | | Ripe berry (12 wpi) | | |
|---|---|---|---|---|---|
| Class | Compounds | LOD(µg/g) | Ctrl | Peg | Eg |
| stilbenoids | astringin | 0.005 | 4.21 ± 0.57 [b] | 13.67 ± 1.52 [a] | 4.85 ± 2.81 [b] |
| | isorhapontin | 0.005 | 0.54 ± 0.04 [b] | 3.33 ± 0.38 [a] | 1.19 ± 0.31 [b] |
| | trans-ε-viniferin | 0.005 | 4.65 ± 0.57 [c] | 41.86 ± 3.82 [a] | 12.12 ± 4 [b] |
| | cis+trans-o-viniferin | 0.0125 | 1.12 ± 0.33 [c] | 22.43 ± 0.01 [a] | 3.28 ± 0.27 [b] |
| | pallidol | 0.005 | 61.04 ± 13.54 [b] | 215.63 ± 5.87 [a] | 68.96 ± 6.62 [b] |
| | ampelopsin D+ quadrangularin A | 0.025 | 0.25 ± 0.04 [c] | 5.49 ± 0.15 [a] | 0.79 ± 0.17 [b] |
| | E-cis-miyabenol | 0.0125 | 0.04 ± 0.03 [c] | 18.58 ± 0.57 [a] | 2.55 ± 0.46 [b] |
| | Z-miyabenol C | 0.05 | 0.04 ± 0.00 [b] | 18.77 ± 5.45 [a] | 1.68 ± 0.35 [b] |
| | isohopeaphenol | 0.0125 | 0.15 ± 0.14 [c] | 132.30 ± 25.67 [a] | 35.84 ± 12.15 [b] |
| | α-viniferin | 0.0125 | 0.01 ± 0.00 [c] | 59.68 ± 6.44 [a] | 10.83 ± 4.03 [b] |
| | ampelopsin H + vaticanol C-like isomer | 0.01 | 0.45 ± 0.35 [c] | 49.83 ± 4.78 [a] | 10.73 ± 2.86 [b] |
| | caffeic acid+catechin condensation | 0.05 | 10.75 ± 1.32 [a] | 1.31 ± 0.24 [b] | 1.31 ± 0.97 [b] |

LOD, limit of detection; Ctrl, control; Peg, pre-egression; Eg, egression; values after ± are standard error. Concentration amounts followed by a common letter are significantly not different between samples, according to Tukey's Honestly Significant Difference test ($P \leq 0.05$), using one way ANOVA

## 3.5. Discussion

Grapevine flowers were challenged by placing suspension of *B. cinerea* conidia, to induce infection. Following that, fungal genes encoding known virulence factors and proteins contribute to the infection program were highly upregulated, as described in Chapter 2. Consequently, the flowers reprogrammed their transcriptome which resulted in increased expression levels of genes involved in reduction-oxidation process, encoding different families of antimicrobial proteins, and those involved in activating polyphenolic metabolites biosynthesis pathway that brought about the production of phytoalexin and precursors for cell wall toughening. These defense reactions of the flowers were unbreakable, forcing *B. cinerea* into quiescence. To know more on the fate of the fungus, we further inspected the inoculated flower until ripening, with an in-depth look of their molecular communication at hard-green (4 wpi) and at ripe (12 wpi) stages.

In the very early stage of quiescence in the flowers, at 96 hpi, ribosomal genes were prevalent in the *in planta* expressed *B. cinerea* genes (78 of the 574 genes, Supplemental Table S2.9). A similar high proportion of ribosomal genes were also observed in the hard-green berry at 4 wpi, 48 out the 289 *in planta* expressed genes (Supplemental Table S3.6). Nevertheless, there was not

any known virulence-related gene in these 289 genes, which could suggest that pathogenesis was "suspended". Yet, other biological activities help the fungus stay "alive" were being carried out since elongation factors, ATP synthesis, and ATP-dependent molecular functions related genes were transcriptionally active. Glyceraldehyde-3-phosphate dehydrogenase (*Bcin15g02120*) and malate dehydrogenase (*Bcin16g04800*), both are highly abundant inside mycelium (Fernández-Acero et al., 2006; 2007) and also involved in glycolysis and tricarboxylic acid cycle for energy metabolism, were expressed. Also among expressed were an ATP-dependent cell division cycle protein 48 (p97/valosin-containing protein, *Bcin08g03700*) gene, involved in cell cycle and transcriptional regulation (Wang et al., 2004), and a number of ATP-dependent membrane transporter genes. To the question where and how the quiescent *B. cinerea* manages to get energy sources, out of the 34 CAZyme genes expressed at the quiescent stage, 4 of them were glycosidases, which can act on cellulose and hemicellulose of plant cell wall. In addition, the upregulation of plant receptors that mediate signaling in response to *B. cinerea*, *PROTEIN KINASE 1* (Abuqamar et al., 2008), and *WAK 1*, which recognizes cell wall–derived oligogalacturonides, (Brutus et al., 2010), indirectly suggest that the quiescent *B. cinerea* was able to obtain energy sources from the plant to carry out its basal metabolic activity.

Notwithstanding the absence of detectable pathogenic progression of the quiescent *B. cinerea*, fruit transcripts that involved in plant defense at initial infection stage (at bloom) continued to be upregulated at 4 wpi. Such continued activation of defense pathways between appresoria formation and quiescent stages of infection was also reported in unripe green tomato infection by *Colletotrichum gloeosporioides* (Alkan et al., 2014). The expressions of *WRKY33* gene, whose expression correlates with the expression of *PR10* genes in response to defense in grapevine (Merz et al., 2015; Chapter 2), and genes of different families of PR proteins, including PR10, were highly induced in the hard-green berry due to the quiescently present *B. cinerea* (Supplemental Table S3.3). The grapevine *WRKY33* functional homologue *AtWRKY33* has been shown to involve in response to biotic and abiotic stresses (Zheng et al., 2006; Jiang and Deyholos, 2009; Li et al., 2011; Birkenbihl et al., 2012). Five genes, encoding a GDSL lipase, whose expression was not affected during flower infection, were strongly upregulated (up to 25-fold) during quiescent infection. There is no previous report associating the lipases encoded by these genes with defense against pathogen. However, they are annotated for catalyzing acyltransfer or hydrolase reactions with lipid and non-lipid substrates, i.e. they have broad

substrate and may interfere with the fungal activity. Other lipases having similar motif, for example, GDSL Lipase 1, targets fungal cell walls and possesses antimicrobial activity (Oh et al., 2005), was shown to be involved in defense against *Alternaria brassicicola* and *B. cinerea* in *Arabidopsis* in an ET dependent manner regulated by *WRKY33* (Oh et al., 2005; Kwon et al., 2009; Birkenbihl et al., 2012).

The upregulation of osmotin, thaumatin, β-1,3-glucanase, and chitinases of the hard-green berry compelled the quiescent *B. cinerea* to underwent a survival cell wall remodeling. The qPCR assay confirmed that *Bcin11g04800*, a gene encoding chitin deacetylase was highly upregulated during quiescent phase (Figure 3.5). Deacetylation of chitin is a mechanism used by plant pathogens as well as endophytic fungi to protect their cell wall from being attacked by plant chitinases (Deising and Siegrist, 1995; El Gueddari et al., 2002). Depolymerisation of chitin into deacetylated chitosan oligomer avoid binding by plant receptors and the consequential plant immune responses (Petutschnig et al., 2010; Liu et al., 2012). Very recently, Cord-Landwehr and colleagues demonstrated that chitosan oligomer, deacetylated chitin extracted from an endophytic fungus *Pestalotiopsis* sp., elicited no plant immunity in rice cell suspension culture (Cord-Landwehr et al., 2016). Thus, the enzyme might play an important role, particularly during quiescent phase, to reduce the recognition of the quiescent *B. cinerea* from the plant immunity. In addition to chitin deacetylase, other genes encoding glycolipid-anchored surface protein and GPI-anchored cell wall organization Ecm33, which in yeast are linked to cell wall integrity to ensure viability (Pardo et al., 2004), were also expressed during the quiescent phase, suggesting that the fungus is also actively defending itself besides the basal metabolic activity.

The activation of stilbenoid and flavonoid biosynthetic pathways by grapevine in response to active pathogenic infection is well documented (Langcake, 1981; Jeandet et al., 1995; Keller et al., 2003; Agudelo-Romero et al., 2015; Kelloniemi et al., 2015; Chapter 2). Here we observed that the genes encoding essential enzymes of the pathways, such as stilbene synthase, chalcone synthase, flavanone 3-hydroxylase, and anthocyanidin 3-O-glucosyltransferase, were actively engaged during quiescent infection. The transcription factor regulating stilbene biosynthesis, MYB14 (Höll et al., 2013) was also modulated. Polyphenols, as measured by HPLC-DAD-MS, too, were found at higher concentration in the inoculated samples. Nonetheless, compared to the infection initiation state at bloom (12-96 hpi), the concentration of the polyphenols was less at 4

wpi in the hard-green berry. The content of resveratrol and its monomeric (for example astringin, isorhapontin, and piceide) and oligmeric (for example miyabenol, isohopeaphenol, and viniferin) derivatives, which are known defense compounds (Langcake, 1981; Pezet, et al., 2003a; Favaron et al., 2007; Hammerbacher et al., 2011), was higher in hard-green berry with quiescent *Botrytis* than control.

Relevant transcripts for the synthesis of monolignols precursors (PAL, COMT, CCoAMT), which increase penetration resistance of the plant tissues (Bhuiyan et al., 2009), and other lignin forming enzymes like GLP3 and EXT, were also induced in the hard-green berry. The result from initial infection in the flower suggested that lignification at the site of penetration as one of the major defense mechanism grapevine flowers use to stop *B. cinerea* progress. The activation of monolignol biosynthetic pathway and the upregulation of GLP3 and EXT together with $H_2O_2$, accumulation that we observed at the site of penetration in the flower were similar mechanisms that véraison berry and green tomatoes employed to stop *B. cinerea* growth (Cantu et al., 2009; Kelloniemi et al., 2015). It is interesting to observe the pathway being active at 4 wpi in the hard-green berry.

The ability of the grape berries to keep the pathogen under quiescence is however broken at ripening (Figure 3.1C). Egression of *B. cinerea* was observed after bagging bunches for two weeks starting at full coloring (approximately 10 wpi), to create high humidity around the bunch. Creating similar high humidity around the bunch, before full coloring, was shown causing no *B. cinerea* egression (Supplemental Figure S2.6). At the very start of the egression process, an outgrowth of *B. cinerea* (or egression) was observed on about 40 % of the berries. Basically, at ripe stage, a single berry with active *B. cinerea* growth could be enough to cause rot on the whole bunch; it will be just a matter of time as the tissue is senescing and therefore its susceptibility to pathogen increases (Kretschmer et al., 2007; Prusky et al., 2013). Since it gets confusing with time to identify berries with "truly" egressed *B. cinerea* or progression thereof around the egression spot, samples were immediately collected to avoid disease progression into asymptomatic spots. As per the state of the fungus in berries that showed no visible symptom/sign (pre-egressed *B. cinerea*), the "initiation" of virulence-related genes, like cerato-platanin *BcSPL1*, required for full virulence (Frías et al., 2011, 2014), that were not expressed in the hard-green berry signaled that the state of the pre-egressed *B. cinerea* in ripe berries was different from that of the quiescent one in the hard-green berry.

During egression about 86 % of the *B. cinerea* transcriptome was expressed, and compared to PDB cultured *B. cinerea* 3,548 of the genes were differentially expressed during egression at ripening. These genes encompasses functional annotations of ROS producers and scavengers, CWDE, proteases, and phytotoxic secondary metabolites, which are all what *B. cinerea* requires for full pathogenicity (as reviewed in Nakajima and Akutsu, 2014). *Botrytis* transcripts belonging to these functional classes were shown to involve during successful infection of ripe grapevine berries and other hosts (De Cremer et al., 2013; Smith et al., 2014; Kelloniemi et al., 2015). However, the expression level of *BcPG6* and *BcPEL-like1*, pectinases which were extremely upregulated during initial infection at bloom (Figure 2.9), was much less both at ripe and in PDB culture. BcPG6 was shown acting as endo-hydrolases, releasing monomers and dimers of D-galacturonate instead of oligomers unlike other PGs (Kars et al., 2005). In general, as expected, the transcriptional activity of the pathogen was high during egression. Indeed, what made the quiescent *B. cinerea* egress is the trickiest research question of this study. But ripening associated signals are speculated triggering the transition from the prolonged quiescent to egression phase.

The changes in physical and chemical properties occurring at ripening, such as activation of phytohormone biosynthesis, cuticular changes, cell wall loosening, conversion of acids into sugars, and a steadily diminishing of antifungal compounds (both preformed and inducible secondary metabolites) are hypothesized to favor pathogen egression (Prusky, 1996; Prusky et al., 2013). It has been sown that the protective role of berry cuticle on *B. cinerea* infection decreased with ripening (Commenil et al., 1997; Mlikota-Gabler et al., 2003). The structural changes in the cell wall polysaccharides that lead to fruit softening were also reported to cause susceptibility to necrotrophic pathogens at fruit ripening (Cantu et al., 2008). In their study, suppression of the ripening-associated cell wall loosening genes reduced the susceptibility of ripe tomato to *B. cinerea* (Cantu et al., 2008). A shift in plant-hormone synthesis and signaling balance happening during ripening can also trigger fungal pathogenicity factors (Prusky, 1996; Prusky et al., 2013). In grapevine, alongside the decline in resistance during ripening, the sugars and organic acids exudates appearing on the berry surface can stimulate and promote *B. cinerea* outgrowth (Padgett and Morrison, 1990; Pezet et al., 2003b; Kretschmer et al., 2007).

Considering the transcriptional alterations underwent in the ripe berry, as a response to *Botrytis* egression, a wide array of defense responses were noticed, suggesting that the tissue under

colonization “never gives up” rearranging its defense mechanisms. The expression level of genes of PR proteins, secondary metabolites, cell wall modification, and phytohormones (biosynthesis and signaling) were affected. Even though hormonal cross-talks in response to pathogen attack are complex (Ferrari et al., 2003, 2007; Rowe et al., 2010), we observed egression of *Botrytis* activated ET and JA pathways while SA pathway was repressed. Similar interplay between JA and SA in grapevine-*Botrytis* interaction was reported by Kelloniemi et al. (2015): JA pathway being activated during compatible berry-*Botrytis* interaction at ripe and SA pathway during incompatible interaction at véraison. With regard to stilbenic secondary metabolites, surprisingly oligomerization was majorly driven by the presence of the fungus. Oligomerization, according to Pezet et al. (2003a), increases toxicity. The amount of oligomeric resveratrol was very little in control than treated berries (with pre-egressed and egressed *Botrytis*); from the treated berries, lesser being in berries with egressed *Botrytis* than pre-egressed one. The *B. cinerea* LACCASE 2 (encoded by *Bcin14g02510*) that oxidizes resveratrol (Schouten et al., 2002) was extremely upregulated (128 fold) in the egressed *Botrytis*. This could probably be one of the reasons that the concentration of oligomeric resveratrols was significantly lower in berries having immense *Botrytis* growth than pre-egressed one.

Last, the evolution of the berry skin tissue is an important component of the berry-*Botrytis* interaction. It has been noticed that the extent of the expression of cell wall modifying genes increased toward maturity. It is actually a phenological cue that once the seeds are mature, ripening-associated softening kicks off. The differential accumulation of xyloglucan endotransglucosylases, involve in cell wall extensibility (Miedes et al., 2011) and polygalacturonases and pectinases, involved in berry softening (Deytieux-Belleau et al., 2008), are phenomenal during berry ripening (Nunan et al., 2001; Lijavetzky et al., 2012). These cell wall modifying genes were remarkably upregulated in ripe berries with both pre-egressed and egressed *B. cinerea*, suggesting that the fungus used advantage of the onset of the fruit’s cell wall self-disassembly, exploiting endogenous developmental programs to activate its own virulence CWDE. In tomato, the ripening associated genes polygalacturonase and expansin have been shown to facilitate susceptibility to *B. cinerea* (Cantu et al., 2008, 2009). It has also been suggested that the fungus can induce unripe fruit’s cell wall-modifying proteins to initiate the induction of susceptibility (Cantu et al., 2009). We, however, haven’t observed any hastening of

ripening process in *Botrytis*-inoculated samples. Both mock- and *Botrytis*-inoculated bunches ripen on similar time after inoculation.

On the other side of cell wall modification, the downregulation of genes encoding GLP3, involved in cell wall strengthening by oxidative cross-linking of cell wall components (Bradley et al., 1992; Godfrey et al., 2007), together with the non-differential regulation of monolignol genes as CCoAMT and F5H, involved in cell wall apposition in response to pathogen attack (Busam et al., 1997; Bhuiyan et al., 2009), strongly suggest that cell wall fortification was not involved.

Taken together cell wall strengthening process is the most missed component from the defense responses at ripening.

## 3.6. Conclusions

The *B. cinerea* inoculated at bloom was quiescent for 12 weeks and egressed at ripening, suggesting that the defense responses of the berries were efficient to halt the growth of the fungus only until maturity. Our study revealed that the defense responses of immature berries (at 4 wpi) that put *B. cinerea* quiescent involved different classes of PR proteins and increased activities of the flavonoid, stilbenoid, and monolignol biosynthesis pathways, for phytoalexins and cell wall strengthening. These responses are similar to the responses of the plant to the pathogen at bloom. In this period, the fungus had cryptic interaction with the berry keeping its basal metabolic activities and deacetylating its cell wall. However, at ripe (at 12 wpi) the pathogen managed to egress and cause bunch rot, using the advantage of the fruit's cell wall self-disassembly and fulfilment of other conditions (including humidity). Consequently, there were different defense responses except cell wall strengthening by the ripe berries, but futile and did not keep the pathogen from its advancement to colonize the berries. The study seems to indicate that, in grapevine-*Botrytis* interaction, cell wall associated defense is principal in keeping *B. cinerea* quiescent.

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## 3.8. Supplemental materials

### 3.8.1. Supplemental tables

Supplemental Table S3.1. List of qPCR primers. Gene identification, primer name, primer sequence, and source are listed.

| | Gene ID | Primer name | Primer sequence | Reference |
|---|---|---|---|---|
| *Botrytis cinerea* | Bcin01g09620 (*BcRPL5*) | Bcrpl5-F | GATGAGACCGTCAAATGGTTC | Zhang and vanKan, 2013 |
| | | Bcrpl5-R | CAGAAGCCCACGTTACGACA | |
| | Bcin01g08040 (*BcTUBA*) | BctubA-F | TTTGGAGCCAGGTACCATGG | Mehli *et al.* 2005 |
| | | BctubA-R | GTCGGGACGGAAGAGTTGAC | |
| | Bcin01g09570 | Bcyt521-F | GTTGGAACTGTCGGAGGTGT | This study |
| | | Bcyt521-R | GCCTTTTCATCAGCTGCTTT | |
| | Bcin02g06140 | BcCP2-F | CGGATTCCCATCATTCAATC | This study |
| | | BcCP2-R | AGGAGGTCCCATTCCCATAC | |
| | Bcin03g01920 | BcCAT-F | CAATGGTCCACTCCTTCTTCA | This study |
| | | BcCAT-R | AACACGCTCTGGGATACGTT | |
| | Bcin07g01540 | BcEF2-F | CTTTGGGTGACGTCCAAGTT | This study |
| | | BcEF2-R | CACCGAATTTCTTGGCGTAT | |
| | Bcin08g05540 | BcCND1-F | GCTACCGATGGTCTTGGAAA | This study |
| | | BcCND1-R | ATCAGTGGTGACGATGTGGA | |
| | Bcin11g04800 | BcCDA-F | CTTCCCTGTTGTTCCTCCAA | This study |
| | | BcCDA-R | TGTCTCATCCGGACTCACAA | |
| | Bcin12g06170 | Bcalle-F | CGAAGTCCTTCCAGAGCAAC | This study |
| | | Bcalle-R | TGGGTCTCTCCGACAGTTCT | |
| | Bcin13g05810 | BcALD-F | ACGGAAAGGCAATCTCAATG | This study |
| | | BcALD-R | CTTATCTGCCCATCCACCAT | |
| | Bcin14g04260 | BcMAS1-F | CGATGGAACTCCTCGTGATT | This study |
| | | BcMAS1-R | GATCATCTTGGCAGCACTGA | |
| *Vitis vinifera* | VIT_04s0044g00580 (*VvACT*) | VvACT-F | ATGTGCCTGCCATGTATGTTGCC | Bèzier *et al.* 2002 |
| | | VvACT-R | AGCTGCTCTTTGCAGTTTCCAGC | |
| | VIT_06s0004g00480 (*VvTUB*) | VvTUB-F | TGTTGGTGAAGGCATGGAGG | Giacomelli *et al.* 2012 |
| | | VvTUB-R | AGATGACACGCCTGCTGAACT | |
| | VIT_01s0010g02020 | VvPER-F | AGGGCAAGCAAGATGTGTGA | This study |
| | | VvPER-R | TCCAGGGGTGCAAGATTGTC | |
| | VIT_01s0146g00480 | VvJAZ10-F | TCCGAAGAATAATCCGCCGT | This study |
| | | VvJAZ10-R | CAGGACTGTAAACCGGCAAC | |
| | VIT_02s0025g01670 | Vv02-F | ACAAGGAAGCAAAAGGAGCA | This study |
| | | Vv02-R | CCTCAGCAAGTTCAACCACA | |
| | VIT_03s0038g03130 | VvFMO3-F | CTCCAAACACCCAAACCATT | This study |
| | | VvFMO3-R | TGAACTTGATGTGCCTGAGC | |
| | VIT_04s0023g03230 | VvSAUR9-F | TGGGAAGAAACCTGGCTATG | This study |
| | | VvSAUR9-R | GGTGAGCCAACCATGAGATT | |
| | VIT_05s0094g00360 | VvCHIT4c-F | TCGAATGCGATGGTGGAAA | Ramírez-Suero *et al.* 2014 |
| | | VvCHIT4c-R | TCCCCTGTCGAAACACCAAG | |
| | VIT_07s0005g03340 | VvMYB14-F | TCTGAGGCCGGATATCAAAC | Höll *et al.* 2013 |
| | | VvMYB14-R | GGGACGCATCAAGAGAGTGT | |
| | VIT_08s0058g00690 | VvWRKY33-F | ATTCAAGCACTAGTATGAACAGAGCAG | Merz *et al.* 2015 |
| | | VvWRKY33-R | CCTTGTTGCCTTGGCATGA | |

Supplemental Table S3.1.Continued.

| | Gene ID | Primer name | Primer sequence | Reference |
|---|---|---|---|---|
| *Vitis vinifera* | VIT_08s0058g00790 | VvSIRD-F | TGAAGACACACTCGGAGCTG | This study |
| | | VvSIRD-R | AGGATATCGAGGCGTCCGTA | |
| | VIT_11s0052g01110 | Vv4CL-F | TTCCCGACATCAACATCCCG | This study |
| | | Vv4CL-R | TTACGTGCGGTGAGATGGAC | |
| | VIT_12s0059g01250 | VvbGlu-F | GCGTTTCTCCTGACCGTCTA | This study |
| | | VvbGlu-R | GCTCTTCATGGCCAACTGAC | |
| | VIT_13s0074g00390 | VvCYP-F | ATCGAAAAGGGCCAGTCTTT | This study |
| | | VvCYP-R | GCATGTTTTGCACCATGTTC | |
| | VIT_14s0066g01150 | VvCCR-F | AGCAGAAACAGGGATGCCAT | This study |
| | | VvCCR-R | AGAGAGCCTCCCATCTGACA | |
| | VIT_14s0171g00180 | VvLRR-F | GCACTGGAAATTTGGGAGAA | This study |
| | | VvLRR-R | GCAAATCCGCTGAAATCACT | |
| | VIT_17s0000g09770 | VvCP-F | GGTTTTGGTTGTTGGGATTG | This study |
| | | VvCP-R | TAAAGCGCTTGTGCTTCTCA | |
| | VIT_18s0001g14790 | VvEXL3-F | GGCGCGAAGAATAGTTGTTG | This study |
| | | VvEXL3-R | GATCCTCGGCACACTCTCTT | |
| | VIT_19s0014g01780 | VvBZIP-F | TTCTTGGGTGGACGAGTTTC | This study |
| | | VvBZIP-R | ATGCCTAGCTTCCTCAACCA | |
| | VIT_19s0093g00320 | VvGST1-F | CCAAAGAGCAAAAGCCAAGT | Conn *et al.* 2008 |
| | | VvGST1-R | TGTCCAGAAAACCCAAAGTC | |

Supplemental Table S3.2. Summary of reads mapping of the 18 RNA-seq libraries Ctrl, mock inoculated; Trt, B. cinerea inoculated; Bc, *Botrytis cinerea*; HG, hard-green berry; Peg, pre-egression; Eg, egression; 1-3 indicate the three biological replicates.

| Library | Total quality-trimmed reads | Reads mapped to *V. vinifera* reference | Reads uniquely mapped to *V. vinifera* reference | Reads mapped to *B. cinerea* reference | Reads uniquely mapped to *B. cinerea* reference |
|---|---|---|---|---|---|
| HG_Ctrl1 | 18,764,162 | 17,226,186 (91.80 %) | 16,552,848 (88.22 %) | 46,268 (0.25 %) | 520 (0.01 %) |
| HG_Ctrl2 | 18,245,810 | 13,462,443 (73.78 %) | 10,041,596 (55.04 %) | 1,675,246 (9.18 %) | 1,786 (0.00 %) |
| HG_Ctrl3 | 20,330,170 | 17,125,573 (84.24 %) | 16,376,294 (80.55 %) | 68,337 (0.34 %) | 545 (0.00 %) |
| HG_Trt1 | 23,828,415 | 21,594,466 (90.62 %) | 20,837,836 (87.45 %) | 45,065 (0.19 %) | 6,958 (0.03 %) |
| HG_Trt2 | 21,976,001 | 19,853,734 (90.34 %) | 19,120,028 (87.00 %) | 56,664 (0.26 %) | 16,928 (0.08 %) |
| HG_Trt3 | 21,146,332 | 18,828,955 (89.04 %) | 18,070,467 (85.45 %) | 75,234 (0.35 %) | 24,680 (0.12 %) |
| Ripe_Ctrl1 | 24,635,902 | 22,048,745 (89.50 %) | 21,007,364 (85.27 %) | 37,989 (0.15 %) | 818 (0.00 %) |
| Ripe_Ctrl2 | 26,843,805 | 24,151,620 (89.97 %) | 23,069,555 (85.94 %) | 27,883 (0.10 %) | 836 (0.00 %) |
| Ripe_Ctrl3 | 29,860,937 | 26,527,044 (88.84 %) | 25,136,297 (84.18 %) | 169,478 (0.57 %) | 885 (0.00 %) |
| Ripe_Eg1 | 21,296,699 | 3,254,793 (15.28 %) | 2,756,774 (12.94 %) | 16,664,468 (78.25 %) | 14,235,574 (66.84 %) |
| Ripe_Eg2 | 22,578,478 | 5,287,093 (23.42 %) | 4,550,569 (20.15 %) | 14,815,131 (65.62 %) | 12,490,580 (55.32 %) |
| Ripe_Eg3 | 28,787,397 | 12,703,484 (44.13 %) | 11,577,654 (40.22 %) | 12,750,536 (44.29 %) | 10,718,882 (37.23 %) |
| Ripe_Peg1 | 45,369,750 | 32,808,151 (72.31 %) | 27,381,446 (60.35 %) | 2,547,394 (5.61 %) | 22,842 (0.05 %) |
| Ripe_Peg2 | 55,880,939 | 45,269,916 (81.01 %) | 42,976,225 (76.91 %) | 2,315,554 (4.14 %) | 1,698,079 (3.04 %) |
| Ripe_Peg3 | 47,239,407 | 40,062,424 (84.81 %) | 38,243,440 (80.96 %) | 118,269 (0.25 %) | 23,815 (0.05 %) |
| Bc1 | 22,223,388 | 76,740 (0.35 %) | 21,423 (0.01 %) | 20,072,229 (90.32 %) | 14,108,503 (63.48 %) |
| Bc2 | 21,289,297 | 47,738 (0.22 %) | 28,559 (0.13 %) | 19,256,732 (90.45 %) | 16,603,159 (77.99 %) |
| Bc3 | 22,254,222 | 50,719 (0.23 %) | 17,798 (0.08 %) | 20,086,452 (90.25 %) | 16,522,732 (74.24 %) |

Supplemental Table S3.8. List of genes used to validate the RNA-Seq expression values by q-PCR assay. Gene identification, gene description, and fold change from RNA-seq and qPCR are provided. HG, har- green berry; Trt, B. Cinerea inoculated; Ctrl, mocj inoculated; Peg, pre-egression; Eg, egression.

| | RNAseq (log2 fold change) | | | | qPCR (log2 fold chnage) | | | |
|---|---|---|---|---|---|---|---|---|
| Gene ID | HG (Trt vs Ctrl) | Ripe (Peg vs ctrl) | Ripe (Eg vs Ctrl) | Ripe (Eg vs peg) | HG (Trt vs Ctrl) | Ripe (Peg vs ctrl) | Ripe (Eg vs Ctrl) | Ripe (Eg vs peg) |
| VIT_01s0010g02020 | 0.03 | 7.60 | 10.83 | 3.23 | 0.06 | 8.36 | 10.49 | 2.14 |
| VIT_01s0146g00480 | 0.80 | 4.84 | 7.36 | 2.52 | 1.38 | 4.05 | 5.91 | 1.86 |
| VIT_02s0025g01670 | 0.56 | -2.26 | -3.51 | -1.25 | 0.12 | -2.16 | -2.77 | -0.61 |
| VIT_03s0038g03130 | -3.29 | 0.04 | 0.02 | -0.02 | -2.35 | -0.71 | -0.40 | 0.31 |
| VIT_04s0023g03230 | -0.03 | -3.05 | -4.92 | -1.87 | 0.16 | -2.68 | -3.70 | -1.02 |
| VIT_05s0094g00360 | 1.84 | 3.38 | 5.12 | 1.75 | 1.41 | 2.98 | 4.27 | 1.29 |
| VIT_07s0005g03340 | 2.22 | 2.37 | 4.52 | 2.16 | 1.12 | 1.94 | 3.48 | 1.53 |
| VIT_08s0058g00690 | 1.61 | 2.17 | 3.69 | 1.52 | 1.23 | 1.42 | 2.69 | 1.27 |
| VIT_08s0058g00790 | 3.23 | 2.78 | 2.55 | -0.23 | 1.52 | 2.18 | 2.23 | 0.04 |
| VIT_11s0052g01110 | 2.69 | 5.02 | 6.12 | 1.10 | 1.42 | 4.15 | 4.73 | 0.58 |
| VIT_12s0059g01250 | -0.10 | -3.70 | -2.68 | 1.02 | -0.09 | -2.62 | -1.47 | 1.15 |
| VIT_13s0074g00390 | -0.44 | -5.48 | -4.76 | 0.72 | -0.29 | -6.58 | -5.86 | 0.72 |
| VIT_14s0066g01150 | 1.05 | 2.85 | 5.02 | 2.17 | 0.60 | 2.28 | 3.76 | 1.48 |
| VIT_14s0171g00180 | -0.45 | -2.75 | -3.05 | -0.30 | -0.44 | -2.40 | -3.13 | -0.74 |
| VIT_17s0000g09770 | -2.00 | 3.83 | 3.04 | -0.79 | -1.56 | 1.78 | 1.55 | -0.23 |
| VIT_18s0001g14790 | 0.30 | -6.42 | -6.29 | 0.14 | 0.27 | -4.32 | -5.53 | -1.21 |
| VIT_19s0014g01780 | -1.25 | -2.44 | -3.18 | -0.74 | -0.70 | -2.20 | -2.46 | -0.26 |
| VIT_19s0093g00320 | 0.03 | 3.82 | 9.04 | 5.21 | 0.07 | 4.02 | 7.79 | 3.78 |

Supplemental Table S3.10. Gene ontology terms enriched in the differentially expressed grapevine genes upon quiescent B. cinerea infection on hard-green berry, 4 wpi. Enriched GO terms are presented. BP, biological process; MF, molecular function; CC, cellular component; wpi, week post inoculation.

| GO term | Ontology | Description | Number in input list | Number in reference list | p-value | FDR |
|---|---|---|---|---|---|---|
| GO:0006950 | BP | response to stress | 68 | 1715 | 2.50E-05 | 4.10E-04 |
| GO:0009856 | BP | pollination | 28 | 203 | 6.00E-14 | 3.20E-12 |
| GO:0006464 | BP | protein modification process | 97 | 2097 | 1.00E-09 | 2.70E-08 |
| GO:0000003 | BP | reproduction | 32 | 439 | 2.00E-08 | 4.80E-07 |
| GO:0019748 | BP | secondary metabolic process | 28 | 374 | 8.10E-08 | 1.70E-06 |
| GO:0006350 | BP | transcription | 74 | 1857 | 1.10E-05 | 1.90E-04 |
| GO:0009056 | BP | catabolic process | 44 | 1084 | 0.00031 | 4.20E-03 |
| GO:0030246 | MF | carbohydrate binding | 39 | 403 | 1.50E-13 | 1.00E-11 |
| GO:0004871 | MF | signal transducer activity | 77 | 1475 | 2.40E-10 | 4.00E-09 |
| GO:0016301 | MF | kinase activity | 100 | 2132 | 3.00E-10 | 4.00E-09 |
| GO:0016740 | MF | transferase activity | 170 | 4541 | 2.00E-08 | 1.90E-07 |
| GO:0003700 | MF | transcription factor activity | 35 | 551 | 1.40E-07 | 1.10E-06 |
| GO:0030528 | MF | transcription regulator activity | 39 | 858 | 7.10E-05 | 5.30E-04 |
| GO:0005576 | CC | extracellular region | 45 | 887 | 1.50E-06 | 1.40E-04 |

Supplemental Table S3.11. Concentration of polyphenolic secondary metabolites in hard-green berry, mock inoculated (Ctrl) or with quiescent B. cinerea (Trt) at 4 wpi. The amount is given in µg/g fresh weight. p-value < 0.05, using unpaired heteroscedastic Student's t test, is highlited. LOD, limit of detection; wpi, week post inoculation; SE, standard error.

| Classe | Secondary metabolites | LOD(µg/g) | Ctrl | | Trt | | |
|---|---|---|---|---|---|---|---|
| | | | Mean | SE | Mean | SE | p-value |
| benzoic acids | *p*-hydroxybenzoic acid | 0.005 | 1.00 | 0.06 | 1.15 | 0.18 | 3.96E-01 |
| | vanillic acid | 0.0025 | 0.01 | 0.00 | 0.01 | 0.00 | 2.64E-01 |
| | gallic acid | 0.025 | 0.11 | 0.02 | 0.16 | 0.06 | 4.03E-01 |
| phenylpropanoids | fraxin | 0.0025 | 0.01 | 0.00 | 0.00 | 0.00 | 3.42E-01 |
| | caftaric acid | 0.0125 | 595.38 | 38.89 | 660.14 | 93.16 | 5.08E-01 |
| | caffeic acid | 0.02 | 0.15 | 0.02 | 0.12 | 0.02 | 4.52E-01 |
| | fertaric acid | 0.0025 | 7.85 | 0.73 | 18.44 | 4.15 | 2.50E-02 |
| | *trans*-coutaric acid | 0.025 | 260.24 | 16.25 | 272.70 | 31.48 | 7.19E-01 |
| dihydrochalcones | phlorizin | 0.0025 | 1.40 | 0.25 | 1.40 | 0.12 | 9.95E-01 |
| flavonoids | luteolin | 0.0025 | 0.01 | 0.00 | 0.04 | 0.02 | 1.30E-01 |
| | luteolin-7-O-Glc | 0.0025 | 0.22 | 0.01 | 0.40 | 0.08 | 3.30E-02 |
| | naringenin | 0.0025 | 0.01 | 0.00 | 0.08 | 0.01 | 1.00E-03 |
| | catechin | 0.025 | 17.75 | 1.23 | 26.06 | 3.88 | 5.90E-02 |
| | epicatechin | 0.025 | 4.40 | 0.44 | 9.33 | 1.19 | 3.00E-02 |
| | epigallocatechin | 1.25 | 2.18 | 0.52 | 1.64 | 0.39 | 4.44E-01 |
| | gallocatechin | 0.25 | 55.54 | 10.82 | 47.12 | 4.59 | 5.06E-01 |
| | epigallocatechin gallate | 0.25 | 0.23 | 0.05 | 0.47 | 0.17 | 2.79E-01 |
| | epicatechin gallate | 0.025 | 0.10 | 0.02 | 1.67 | 0.29 | 1.30E-02 |
| | procyanidin B1 | 0.0125 | 67.98 | 4.47 | 136.63 | 28.81 | 1.00E-01 |
| | procyanidin B2 + B4 | 0.125 | 2.52 | 0.49 | 3.74 | 0.64 | 1.79E-01 |
| | procyanidin B3 (as B1) | 0.0125 | 25.27 | 1.28 | 47.21 | 9.14 | 9.80E-02 |
| | taxifolin | 0.0025 | 6.54 | 0.95 | 13.40 | 3.46 | 1.52E-01 |
| | myricetin | 1.25 | 1.22 | 0.03 | 1.30 | 0.16 | 6.79E-01 |
| | Quercetin-3-Rha | 0.005 | 0.04 | 0.01 | 0.06 | 0.02 | 3.06E-01 |
| | kaempferol-3-Glc | 0.0025 | 0.02 | 0.01 | 0.08 | 0.03 | 1.62E-01 |
| | quercetin-3-Glc+quercetin-3-Gal | 0.0025 | 4.17 | 0.28 | 6.82 | 1.26 | 1.32E-01 |
| | isorhamnetin-3-Glc | 0.0025 | 0.09 | 0.01 | 0.14 | 0.02 | 7.10E-02 |
| | kaempferol-3-rutinoside | 0.005 | 0.03 | 0.00 | 0.07 | 0.02 | 1.89E-01 |
| | Quercetin-3-Glc-Ara | 0.005 | 0.02 | 0.00 | 0.05 | 0.01 | 9.90E-02 |
| | rutin | 0.0025 | 1.50 | 0.22 | 2.40 | 0.47 | 1.56E-01 |
| | isorhamnetin-3-rutinoside | 0.005 | n.d. | | 0.02 | 0.01 | |
| | Quercetin-3-glucuronide | 0.0125 | 27.21 | 2.83 | 36.84 | 7.30 | 3.06E-01 |
| | Kaempferol-3-glucuronide | 0.005 | 0.13 | 0.03 | 0.24 | 0.04 | 7.50E-02 |
| stilbenoids | arbutin | 0.005 | 0.13 | 0.01 | 0.90 | 0.19 | 2.70E-02 |
| | *trans*-resveratrol | 0.005 | n.d. | | 1.52 | 0.47 | |
| | *cis*-resveratrol | 0.0025 | n.d. | | 0.01 | 0.00 | |
| | piceatannol | 0.0025 | 0.01 | 0.00 | 0.19 | 0.08 | 1.18E-01 |
| | *trans*-piceide | 0.0125 | 0.97 | 0.09 | 10.08 | 1.83 | 1.60E-02 |
| | *cis*-piceide | 0.0025 | 0.34 | 0.05 | 1.70 | 0.40 | 4.20E-02 |
| | astringin | 0.005 | 0.14 | 0.03 | 1.55 | 0.26 | 1.20E-02 |
| | isorhapontin | 0.005 | n.d. | | 0.21 | 0.06 | |
| | *trans*-ε-viniferin | 0.005 | n.d. | | 9.86 | 2.16 | |
| | *cis*+*trans*-ο-viniferin | 0.0125 | n.d. | | 0.30 | 0.07 | |
| | caffeic acid+catechin condensation | 0.05 | 2.01 | 0.28 | 4.76 | 1.22 | 1.17E-01 |
| | pallidol | 0.005 | n.d. | | 0.60 | 0.19 | |
| | ampelopsin D+quadrangularin A | 0.025 | n.d. | | 2.78 | 0.81 | |
| | α-viniferin | 0.0125 | n.d. | | 1.86 | 0.66 | |
| | E-*cis*-miyabenol | 0.0125 | n.d. | | 0.91 | 0.34 | |
| | Z-miyabenol C | 0.05 | n.d. | | 0.40 | 0.12 | |
| | isohopeaphenol | 0.0125 | n.d. | | 0.16 | 0.05 | |

Supplemental Table S3.14. Gene ontology terms enriched in the differentially expressed grapevine genes in ripe berry with pre-egressed and egressed B. cinerea at 12 wpi. Enriched GO terms are presented. BP, biological process; MF, molecular function; CC, cellular component; wpi, week post inoculation.

| Stage | GO term | Ontology | Description | Number in input list | Number in reference list | p-value | FDR |
|---|---|---|---|---|---|---|---|
| Pre-egression | GO:0019748 | BP | secondary metabolic process | 102 | 374 | 3.80E-14 | 1.40E-11 |
| | GO:0050896 | BP | response to stimulus | 341 | 2206 | 9.00E-05 | 5.50E-03 |
| | GO:0006810 | BP | transport | 350 | 2301 | 0.00023 | 8.30E-03 |
| | GO:0043412 | BP | macromolecule modification | 363 | 2098 | 1.70E-09 | 2.10E-07 |
| | GO:0006464 | BP | protein modification process | 363 | 2097 | 1.60E-09 | 2.10E-07 |
| | GO:0004871 | MF | signal transducer activity | 295 | 1475 | 6.20E-15 | 2.40E-13 |
| | GO:0016740 | MF | transferase activity | 721 | 4541 | 2.10E-09 | 4.00E-08 |
| | GO:0030246 | MF | carbohydrate binding | 81 | 403 | 1.70E-05 | 2.70E-04 |
| | GO:0005215 | MF | transporter activity | 215 | 1292 | 2.30E-05 | 3.30E-04 |
| | GO:0003824 | MF | catalytic activity | 1652 | 11955 | 0.00058 | 7.30E-03 |
| | GO:0005886 | CC | plasma membrane | 323 | 1654 | 1.00E-14 | 1.80E-12 |
| | GO:0016020 | CC | membrane | 823 | 5038 | 1.80E-12 | 1.60E-10 |
| Egression | GO:0019748 | BP | secondary metabolic process | 135 | 374 | 4.30E-16 | 1.60E-13 |
| | GO:0050896 | BP | response to stimulus | 486 | 2206 | 8.60E-06 | 1.40E-03 |
| | GO:0006950 | BP | response to stress | 385 | 1715 | 1.20E-05 | 1.40E-03 |
| | GO:0006810 | BP | transport | 492 | 2301 | 0.00013 | 6.20E-03 |
| | GO:0009056 | BP | catabolic process | 253 | 1084 | 2.10E-05 | 1.90E-03 |
| | GO:0005975 | BP | carbohydrate metabolic process | 241 | 1063 | 0.0002 | 8.20E-03 |
| | GO:0003700 | MF | transcription factor activity | 128 | 548 | 0.00092 | 2.80E-02 |
| | GO:0005886 | CC | plasma membrane | 421 | 1654 | 1.20E-12 | 2.40E-10 |
| | GO:0016020 | CC | membrane | 1096 | 5038 | 5.00E-09 | 4.80E-07 |
| | GO:0005737 | CC | cytoplasm | 961 | 4644 | 4.70E-05 | 3.00E-03 |
| | GO:0005623 | CC | cell | 2320 | 11670 | 9.20E-05 | 4.40E-03 |
| | GO:0044464 | CC | cell part | 2300 | 11600 | 0.00015 | 5.80E-03 |

Supplemental Table S3.3. Differentially expressed *V. vinifera* genes due to quiescent *B. cinerea* presence on hard-green berry. (Provided as excel file)

Supplemental Table S3.4. Differentially expressed V. *vinifera* genes due to pre-egressed and egressed *B. cinerea* during ripening. (Provided as excel file)

Supplemental Table S3.5. Differentially expressed *Botrytis cinerea* genes during egression on ripe berry as compared to PDB grown *B. cinerea*. (Provided as excel file)

Supplemental Table S3.6. *Botrytis cinerea* genes expressed in planta, in hard-green berry during quescent infection. (Provided as excel file)

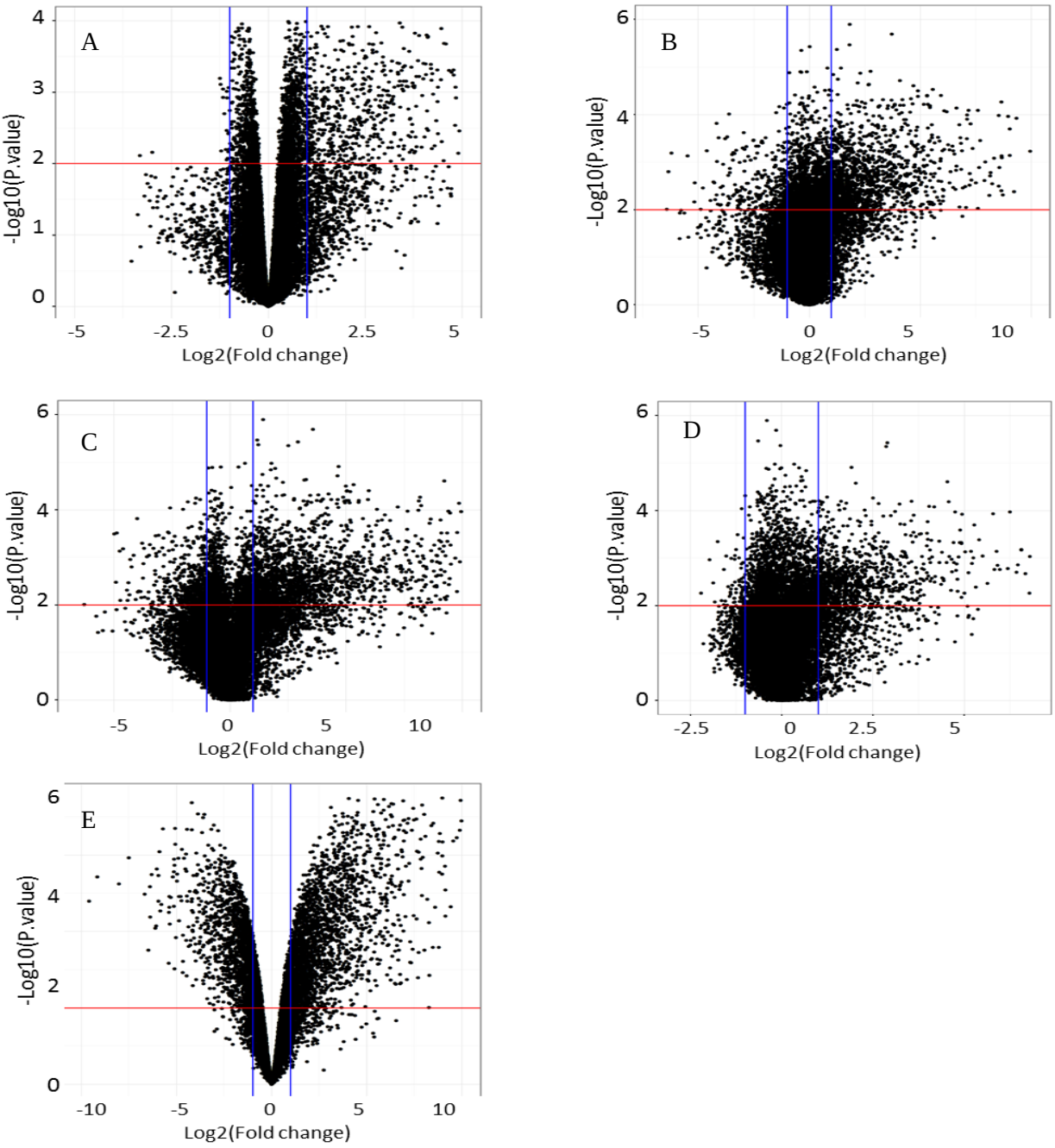
Supplemental Table S3.7. Botrytis cinerea genes expressed in planta, in ripe berry (pre-egressed). (Provided as excel file)

Supplemental Table S3.9. Gene ontology (GO) annotation of quescent B. cinerea genes expressed in planta in hard-green berry stage. (Provided as excel file)

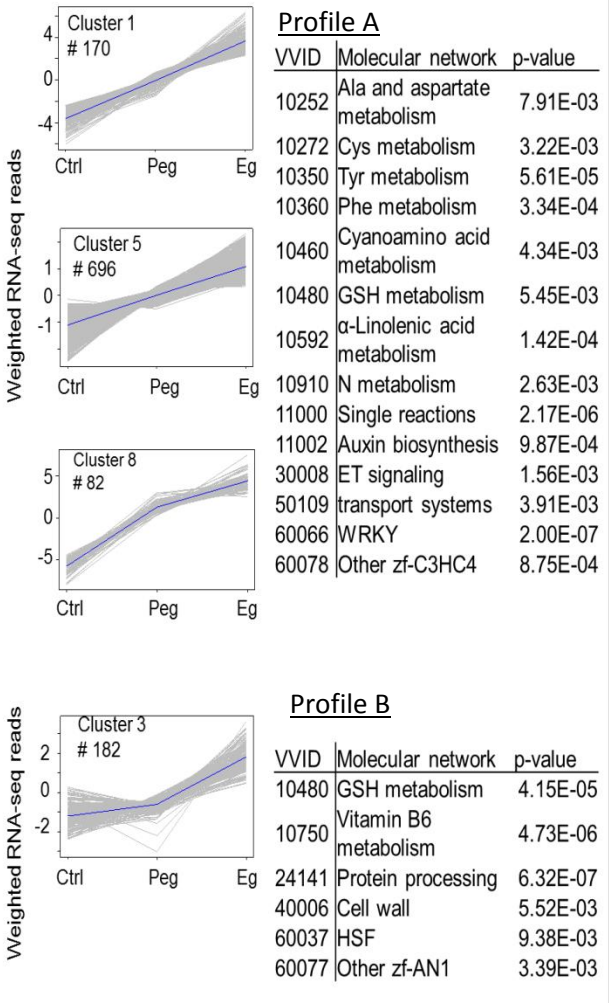
Supplemental Table S3.12. Gene ontology (GO) annotation of in-planta-expressed genes of pre-egressed *B. cinerea* in ripe berry. (Provided as excel file)

Supplemental Table S3.13. Gene ontology (GO) annotation of genes of egressed *B. cinerea* in ripe berry. (Provided as excel file)

### 3.8.2. Supplemental figures




Supplemental Figure S3.1. Volcano plots of grapevine and *B. cinerea* gene expression values. A, har-green berry, Trt vs Ctrl; B, Ripe berry, pre-egressed vs Ctrl; C, Ripe berry, egressed vs Ctrl; D, Ripe berry, egressed vs pre-egressed; and E, *B. cinerea*, egressed vs PDB cultures. Genes in the left top rectangle [log 10(*P.* value) > 2 and log2(fold change) < -1] were selected as downregulated; whereas, genes in the right top rectangle were selected as upregulated [log 10(*P.* value) > 2 and log2(fold change) > 1] .

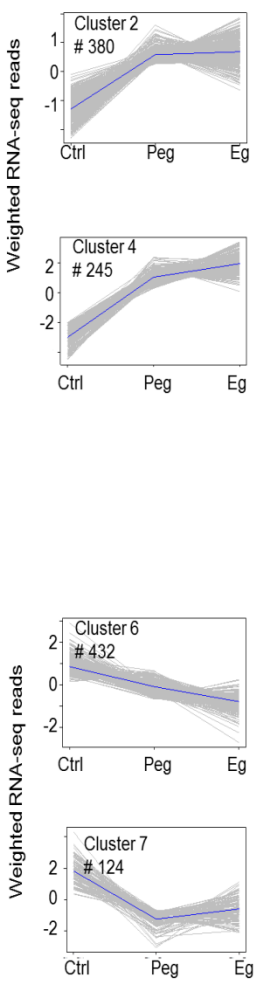



Profile A

| VVID | Molecular network | p-value |
|---|---|---|
| 10252 | Ala and aspartate metabolism | 7.91E-03 |
| 10272 | Cys metabolism | 3.22E-03 |
| 10350 | Tyr metabolism | 5.61E-05 |
| 10360 | Phe metabolism | 3.34E-04 |
| 10460 | Cyanoamino acid metabolism | 4.34E-03 |
| 10480 | GSH metabolism | 5.45E-03 |
| 10592 | α-Linolenic acid metabolism | 1.42E-04 |
| 10910 | N metabolism | 2.63E-03 |
| 11000 | Single reactions | 2.17E-06 |
| 11002 | Auxin biosynthesis | 9.87E-04 |
| 30008 | ET signaling | 1.56E-03 |
| 50109 | transport systems | 3.91E-03 |
| 60066 | WRKY | 2.00E-07 |
| 60078 | Other zf-C3HC4 | 8.75E-04 |

Profile B

| VVID | Molecular network | p-value |
|---|---|---|
| 10480 | GSH metabolism | 4.15E-05 |
| 10750 | Vitamin B6 metabolism | 4.73E-06 |
| 24141 | Protein processing | 6.32E-07 |
| 40006 | Cell wall | 5.52E-03 |
| 60037 | HSF | 9.38E-03 |
| 60077 | Other zf-AN1 | 3.39E-03 |




Profile C

| VVID | Molecular network | p-value |
|---|---|---|
| 10272 | Cys metabolism | 9.33E-04 |
| 10350 | Tyr metabolism | 8.77E-03 |
| 10360 | Phe metabolism | 3.11E-03 |
| 10450 | Selenoamino acid metabolism | 8.74E-03 |
| 10500 | Starch and sucrose metabolism | 6.30E-03 |
| 10530 | Aminosugars metabolism | 6.99E-03 |
| 10592 | α-Linolenic acid metabolism | 1.38E-05 |
| 10920 | Sulfur metabolism | 9.04E-03 |
| 10940 | Phenylpropanoid biosynthesis | 8.31E-15 |
| 30008 | ET signaling | 6.02E-03 |
| 30011 | JA signaling | 2.70E-03 |
| 60066 | WRKY | 4.45E-04 |
| 60069 | ZIM | 2.68E-03 |

Profile D

| VVID | Molecular network | p-value |
|---|---|---|
| 10500 | Starch and sucrose metabolism | 1.01E-04 |
| 23410 | Base excision repair | 4.50E-03 |
| 30003 | Auxin signaling | 2.29E-03 |

Supplemental Figure S3.2. Profiles of grapevine berry trancripts at ripe stage in reponse to pre-egressed and egressed *B. cinerea* inoculation. K-means clustering of grapevine genes based on the Euclidean distance of the weighted RNA-seq reads. Genes that showed at least twofold expression difference with p-value < 0.01 were considered, and clustered into 8 clusters. The clusters fall into 4 major profiles (A, B, C, and D). Molecular enrichment analysis based on VitisNet was provided for each group.

# 4. CONCLUDING REMARKS

The research reported in this thesis investigated the interaction between *B. cinerea* and grapevine inflorescences/berry at infection initiation, entry to quiescence, quiescence, and egression. Confocal microscopy, classical plating out method, and molecular analyses were used on samples inoculated with B05.10 conidia or mock at bloom (EL25/26 grapevine phenological stage).

The increased level of expression of *B. cinerea* virulence related genes involved in plant cell wall degradation, ROS accumulation, and phytotoxins biosynthesis, upon conidial contact with the flower surface, instigated defense responses in the grapevine tissues. The flowers, upon perceiving the threat, reprogrammed the transcriptions of genes: encoding different antimicrobial proteins, involved in ROS accumulation and secondary metabolites biosynthesis pathways, in phytoalexin production and cell wall reinforcement. These physical and chemical defense responses of the grapevine flowers were all together very efficient in blocking *B. cinerea* growth and inducing quiescence. Similar defense mechanisms were also deployed by the hard-green berries, 4 wpi, to keep the pathogen in quiescent state. From the disease management point of view, the quiescent infection is a very important one as the infecting pathogen remains undetected and causes the disease that impairs crop quantity, quality, and appearance when the conditions are right for re-initiating the infection. Depending on the pathogen and host condition, quiescent stage can last from few days to months. In this study, *B. cinerea* underwent 12 weeks of quiescence before it resumed its active growth at ripening.

During egression at ripe, almost all *Botrytis* virulence and growth related genes were expressed. Hence, the fungus was able to cause bunch rot despite the futile defense responses of the berries. From the results, it seems that the fungus perceived and exploited ripening associated physico-chemical changes and favorable external conditions to switch to an active state of growth from its long-lived quiescence state. In berries with egressed *Botrytis*, the expression level of a number of genes involved in cell wall self-disassembly were higher than in the control berries, suggesting that the egressed *Botrytis* exploited the ontogenically activated cell wall self-disassembly genes. Besides, it was also observed that the cell wall strengthening process, used as a physical barrier to block the pathogen from entering into the inner layers of the infected tissue, was not activated. Perhaps, since cell wall self-disassembly is a general physiological cue during fruit ripening, cell wall stiffening might not be used as defense response by ripening fruits.

In conclusion, according to the results obtained, the defense responses of the grapevine flowers and the hard-green berries were able to keep *B. cinerea* under quiescence. However, exploiting the ripening associated physico-chemical changes in the ripe berries, the fungus recovered an active metabolism and pathogenic activity and caused bunch rot at ripe.

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After spending four years at the Fondazione Edmund Mach (FEM), S. Michele, Italy, now is the time to write this note of thanks in the finishing moment of my stay, wrapping up all what I have done in the past 4 years. It has been a very nice period of learning for me, both scientifically and on personal level. I, therefore, would like to reflect on the people who have supported and helped me so much throughout my stay.

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