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A comparative study of plant-pathogen interaction in different genotypes and organs of grapevine (*Vitis* spp.), based on optimized and new screening methods for resistance to downy mildew (*Plasmopara viticola* - Berk. & Curt.)

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Ho domandato, allora: "Signore,
Tu avevi detto che saresti stato con me in tutti i giorni della mia vita,
ed io ho accettato di vivere con te,
perché mi hai lasciato solo proprio nei momenti più difficili?"
Ed il Signore rispose: "Figlio mio, Io ti amo e ti dissi che sarei stato con te
e che non ti avrei lasciato solo neppure per un attimo:
i giorni in cui tu hai visto solo un'orma sulla sabbia,
sono stati i giorni in cui ti ho portato in braccio".
(L’orma sulla sabbia)

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ii) ABSTRACT

The first target of my research project has been to study the organ-specific interaction between grapevine and downy mildew (DM), caused by the biotrofic pathogen *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni. The studied genotypes have been chosen based on their susceptibility, tolerance and resistance to DM according to previous field observations based on the OIV452 and OIV453 descriptors for leaves and inflorescences, respectively. These two organs have been collected from 9 *Vitis* hybrids and a *Vitis vinifera* L. variety grown in an untreated experimental field at Edmund Mach Foundation (FEM). In particular, inflorescences have been harvested at three phenological stages (17, 25 and 29) of the Eichhorn-Lorenz (E-L) scale. Firstly, I have improved the annotation procedure of foliar resistance/susceptibility under controlled conditions (optimized and updated OIV descriptor 452-1). The *in vitro* leaf disc bioassay resulted significantly associated to the in-field DM response. Secondly, I have developed a new *in vitro* phenotyping method - from infection to symptom evaluation - for DM resistance assessment on grapevine inflorescence (developed and proposed OIV descriptor 453-1). The 17 E-L phenological stage of inflorescence upon *in vitro* assay resulted significantly correlated to the in-field DM response. Based on the latter DM response assessment, genotypes have been assigned to classes following the four thresholds set for leaf discs. Linear correlations between organs within the same genotype have confirmed that the E-L 17 stage is the most responsive to the DM attack. Genotypes classified as mid-resistant or resistant at leaf level have also resistant inflorescences; genotypes with mid-susceptible leaves present susceptible inflorescences except for Cabernet Cortis (CC) (Cabernet Sauvignon x Solaris), carrying mid-resistant leaves and mid-susceptible inflorescences.

To verify that the CC had a significantly different response between organs under controlled conditions, I have carried out some experiments detaching organs from fruiting cuttings grown in phytotron. Thus, CC organs were evaluated upon mock- and *P. viticola*-inoculation, using the susceptible Pinot Noir organs as a reference. Unlike the latter, CC presented a significant difference in DM response; this represented an interesting result and corroborates the diverse DM epidemiological behaviour at organ level.

In the second part of my research project, I have used CC as a model to study divergent dual epidemics, compared to its parent Cabernet Sauvignon (CS) displaying out susceptible organs. Leaves and inflorescences – at the 17 E-L phenological stage – obtained from

fruiting cuttings have been used. To assess the response of different grapevine organs to *P. viticola* attack, the disease development has been evaluated with different methods, from the macroscopic/microscopic to the ultrastructural point of view.

Upon *P. viticola* inoculation, the CC and CS organs showed different levels of resistance or susceptibility, according to previous phenotypic observations and organ classification. Indeed, CC leaves were characterized by some limited attack areas with sparse sporulation and necrotic dots, while the CC inflorescences by abundant and localized sporulation. Moreover, CS organs were both characterized by an abundant and widespread sporulation. Afterward, H_2O_2 production was histochemically evaluated by DAB staining. Its accumulation was clearly visible only on the surface of the CC leaves, suggesting that only the mid-resistant organ could be able to activate H_2O_2 production that interferes with pathogen growth and diffusion.

There results have led me to investigate the different interaction between *P. viticola* and two grapevine organs at ultrastructural level. Interestingly, three zones showing different response to *P. viticola*-inoculation in CC leaf tissues were found: the leaf areas in correspondence to pathogen sporulation, the leaf areas corresponding to the border between green and brown spots, and the necrosis zones. CC inflorescence tissues presented two types of pathogen-response zones: areas in which pathogen structures were deformed and the necrosis areas. Conversely, *P. viticola* was typically structured in both CS tissues.

Encouraged by the preliminary results and since the divergent dual epidemics are poorly studied on a molecular point of view, I have decided to perform a trascriptomics study to identify the differential expressed genes (DEGs) in CC organs both at baseline and upon *P. viticola*-inoculation, using CS as a reference. DEGs were grouped in 14 clusters based on their expression profiles. Interestingly, in the mid-resistant leaves of CC, there was an up-regulation of genes related to secondary metabolism, in particular genes belonging to signal transduction, phytoalexin biosynthesis and oxidative stress response. Meanwhile the genes involved in the protein modification and secondary metabolic process were up-regulated in the susceptible leaves of CS. Genes implicated in the secondary metabolism, response to stimuli, protein modification, carbohydrate metabolism and reproduction were up-regulated in the mid-susceptible inflorescences of CC, while only a weak response involving genes of secondary metabolism and response to stimuli was found in the susceptible inflorescences of CS. Among genes down-regulated upon *P. viticola*-inoculation, only in CC inflorescence the biological processes functional were significantly

enriched. This cluster presented a down-regulation of genes involved in photosynthesis, generation of precursor metabolites and energy, and carbohydrate metabolic process suggesting a broad reduction of the photosynthetic process.

Finally, to validate the organ-specific response to *P. viticola*-inoculation, the disease development and the expression level of some DEGs have been evaluated on an independent experiment, using the two studied genotypes, the reference *V. vinifera* variety Pinot noir and the CC-related *Vitis* hybrid Muscaris.

CHAPTER 1

GENERAL INTRODUCTION

Summary

Viticulture arises in the Neolithic and nowadays holds a relevant role in the worldwide economy. The cultivated *Vitis vinifera* L. species is highly susceptible to several pathogens that threaten the grape/wine economy. Downy mildew (DM) caused by the obligate biotrophic oomycete *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni is one of the main plagues affecting grapevine. Accidentally introduced in Europe in 1878, it obligates the winemakers to massively use fungicides to protect grapevine against infections and avoid crop yield losses. However, fungicides lead to environmental pollution, development of resistance and residual toxicity and they foster human health concerns. An alternative approach in DM management is represented by the use of biocontrol agents or the development of new varieties with innate disease resistance, obtained from crossing between *V. vinifera* cultivars and resistant/tolerant *Vitis* species. Recently, many fungus-resistant grapevine varieties, possessing desirable agronomic attributes and enological characteristics, have been developed in North America and Europe for conventional and especially organic farming.

The development of the modern “omics” approaches (next generation sequencing/genomics, QTLomics, transcriptomics, proteomics and metabolomics), associated with comparative studies are shedding light into the early host responses to DM attack and into the complex plant defence mechanisms.

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"Breeding for grapevine downy mildew resistance: a review of "omics" approaches"

1.1. GRAPEVINE

1.1.1. Grapevine taxonomy

The family *Vitaceae* belongs to the *Rhamnales* order and includes two subfamilies: *Lecoideae* and *Ampelideae.* The subfamily *Ampelideae* includes five genera: *Ampelopsis*, *Cissus, Parthenocissus, Ampelocissus* and *Vitis.* The first four ones are used such as ornamental plants while the *Vitis* genus comprises species largely cultivated and of considerable economic value. This last genus is further divided in two subgenera: *Muscadinia* and *Euvitis* (Fig. 1.1a), including about 40 Asiatic and 30 American species. One of the most important differences between this two subgenera is the chromosome number, with 2n = 40 for the first and 2n = 38 for the second (Fregoni, 2005).

The *Euvitis* subgenus is divided in four groups and the classification is based on their geographical distribution and optimal climate conditions. Two American groups, one Euro-asiatic and one Eastern Asiatic have been identified. The American grapevines in turn have been divided into group of those adapted to temperate climates and those adapted to tropical one. (Fig. 1.1b) (Fregoni, 2005). Most of the American and Asiatic species of grapevine are resistant to several pathogens but their wines are not so appreciate by the consumers, due to their bad quality. In the Euro-asiatic group, the *Vitis vinifera* L. species is the most important worldwide for qualitative attitudes but unfortunately all varieties are highly susceptible to several pathogens; only very few exceptions are recorded.

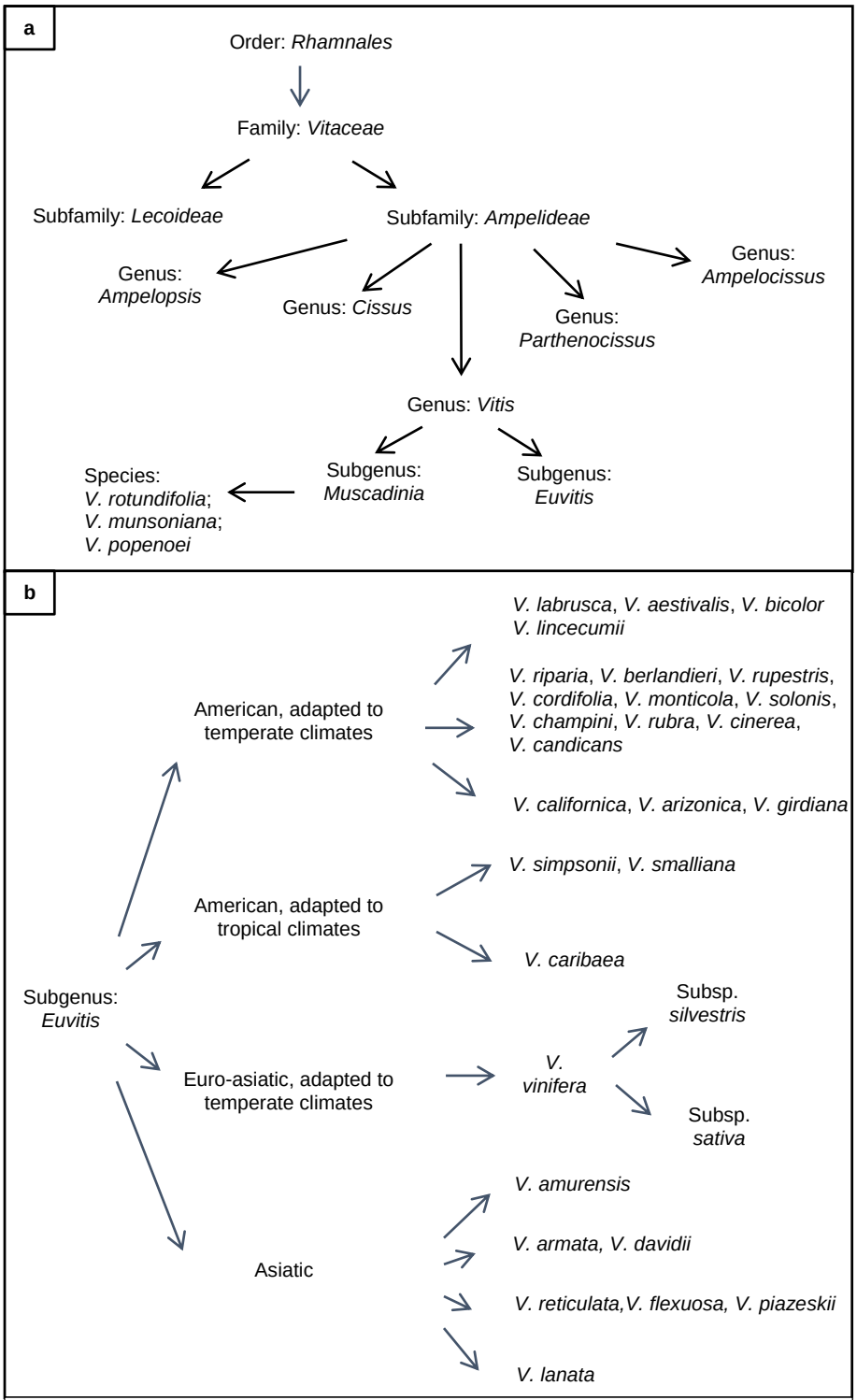

Figure 1.1. An overview on the systematics of the *Vitaceae* family (a) and subgenus *Euvitis* (b) (Adapted from Fregoni, 2005).

1.1.2. Viticulture and its economic importance

Originally, the grapevine and the viticulture had separate lives. The *Vitis* genus has three origin centres: northern America, Asia and Euro-asiatic; its presence in North America indicates that evolution of this genus predates the continental drift occurred about sixty five million years ago.

Viticulture – namely the cultivation of grapevine – arises in to Neolithic with varieties selection (denominated “cultivar”) carried by the Caucasian population. After the last glaciation, it spreads from Black Sea to Egypt, Mediterranean and northern Europe (Fregoni 2005). The cultivated European varieties of *V. vinifera* have Asiatic origins, even though it is still argued whether the differences between the two are the result of geographical isolation or rather of domestication. Since the beginning of domestication, selection has been a powerful tool for enhancing desirable traits according to the final purpose requirements and as a consequence grapevine biology has undergone several drastic changes, for example in berries size, in sugar content, in seed morphology and in flower sexual traits (This et al. 2006). A comprehensive case could be the presence of seedless varieties in many modern table grapes; in fact, this trait impairs the plant to reproduce and thus brings the species to high risk of extinction. Such a character would have never evolved without the assistance of human interference.

Wine is important in many ancient cultures of the human history. Wine making tradition was probably born in the Asia Minor and Greece, from where it expanded throughout the Mediterranean, possibly following the ancient Phoenician trading sea routes, as the distribution of the classic European wine producing areas along river valleys suggests (Robinson 2007). During the Middle Ages, the viticulture suffered the consequences of the social, economic and political crisis through the state of abandon of the farmlands. The grapevines were maintained and cultivated close to churches and monasteries for wine production due to the role that it holds in the catholic ceremonies. From the Renaissance onwards, there has been an incessant recovery of the viticulture and of wine consumption (Fregoni, 2005).

Nowadays, grapevine represents a great agricultural and economical value worldwide with 7,534 million Ha invested in viticulture; Spain, China and France are the countries with the higher number Ha of vineyards. In 2015, a total wine production of 274,4 million Hl has been documented, and Italy is the first producer worldwide, followed by France, Spain and USA. After years of reduction, the wine consumption stabilises at 240 million HI, especially in the American market. The oenological exchanges are stabilised at

104,3 million HI and the total business value is equal to 28,3 billion € (OIV report 2015, http://www.oiv.int). In addition to the wine production and table grapes, there are many derivatives and commodities that are consumed, amongst all grape juices, raisins, currants, brandy, “fortified” wines (port or sherry), distillates and vinegar.

1.1.3. Grapevine pathogenic diseases

During their lifetime, plants are exposed to a wide variety of pathogens, such as bacteria, viruses, fungi and nematodes. According to their lifecycle and infection strategies, pathogenic microorganisms can be classified as necrotrophics, biotrophics and hemibiotrophics. Necrotrophic pathogens feed on dead tissue, secreting lytic enzymes and phytotoxins to promote cell death into the host plant. Conversely, biotrophic pathogens feed on living tissue, developing structures in order to invade the cell and obtain metabolism products. Finally, hemibiotrophic pathogens start with a biotrophic infection phase and then turn to a final necrotrophic phase, killing its host at the end of the infection cycle (Glazebrook 2005). The most cultivated varieties of *V. vinifera* are widely affected by a large number of pathogenic microorganisms that cause several diseases. Among the potential threats, bacteria, fungi, oomycete or viruses can attack grapevine with different infection mechanisms and evasion strategies (Armijo et al. 2016). Some of the most important diseases in *V. vinifera* are the gray mould, powdery mildew, and downy mildew (DM), caused by *Botrytis cinerea*, *Erysiphe necator* and *Plasmopara viticola*, respectively. DM was accidentally introduced in Europe in 1860 through the importation of resistant American vines to *Phylloxera vitifoliae,* a root aphid that brings to wilt and death of grapevines. The plague spread from France to Italy and subsequently all across Europe, reaching the African coasts as well (Galet 1977), destroying the vineyards and producing considerable yield loss.

1.1.3.1. Gray mould

Grapevine gray mould is caused by the necrotrophic ascomycete*, B. cinerea* Pers.Fr., which causes a disease in post-harvest periods, affecting complete berry clusters during packing, transport and commercialization and thus becoming one of the most important pathogens concerning export wine and table grapes (Dean et al. 2012). The fungus has the ability to live as a parasite in green tissue and as a saprophyte in dead or decaying ones, reason for which it is widely distributed in nature (Armijo et al. 2016).

B. cinerea infects grapevine by two main mechanisms: 1) direct mycelium penetration through skin pores or injuries; 2) early invasion, where conidia infect mainly the flower receptacle, and to a lesser extent the stigma and styles, remaining latent within the berry until maturity (Viret et al. 2004). The fungus attaches to a solid host-surface where takes place the conidial germination (Cotoras et al. 2009). The conidium perceives the nutrients from the host surface; it develops an infective structure called appressorium, which breaches the cuticle by means of a penetration peg (Rolke et al., 2004). The appressorium secretes cell wall degrading enzymes (CWDE) to cross cuticle and outer epithelial wall. To promote host cell death and sporulation, *B. cinerea* secretes toxins and oxalic acid during infection (van Kan, 2006). The fungus can overwinter in soil thanks to the production of resistant survival structures (known as sclerotia), which may germinate the next spring, producing conidiophores. Under specific climatic (i.e., moist nights, foggy mornings and dry days) and edaphic (i.e., low nutrient and well-drained soils) conditions, *Botrytis* infections may occur slowly causing “noble rot” (Fig. 1.2a-b) (Ribéreau-Gayon et al., 1980, Blanco-Ulate et al. 2015). This type of infection promotes the accumulation of aroma and flavor compounds as well as the concentration of sugars (Vannini and Chilosi, 2013, Blanco-Ulate et al. 2015), which is generally exploited by winemakers for the production of sweet dessert wines (Rieger 2006).

1.1.3.2. Powdery mildew

Grapevine powdery mildew is caused by the obligate biotrophic ascomycete *Uncinula necator* (syn. *E. necator*), and it is considered to be one of the most important fungal diseases in viticulture worldwide. Symptoms appear as white-grayish powdery or dusty patches of fungus growth on the upper side of the leaves and on other green parts of the vines, leading to a decrease in photosynthetic activity (Fig. 1.2c-d). In infected clusters, berries turn hard, brown, are smaller than uninfected ones, and may split open (Fig. 1.2e) (Gomès and Thévenot, 2009). Besides direct loss of yield, infected berries fail to properly mature and significantly alter wine quality (Gadoury et al. 2001, Calonnec et al. 2004). *E. necator* depends on its host for growth and development. For this, the conidium attaches to the tissue cells of the plant, allowing the formation of a primary germ tube that differentiates into a specialized infectious structure (i.e., appressorium). It generates a mechanical pressure in order to penetrate and invade the host cells (Armijo et al. 2016). The successful invasion results in the haustorium formation, by which the fungus absorbs nutrients necessary to complete its lifecycle (Qiu et al. 2015). Once this structure is established,

secondary hyphae spread along the infected tissue and finally asexual reproductive bodies (i.e., conidiophores and conidia) emerge from them. When environmental or nutritional conditions become unfavourable, *E. necator* develops cleistothecia, structures of sexual reproduction, which contain from four to six asci at maturity, each of which usually contains four ascospores (Agrios 1997, Armijo et al. 2016). However, physiological maturity may not be reached for several months, particularly in colder climates. Like conidia, ascospores germinate with a single germ tube, which terminates in appressorium formation (Gadoury et al., 2012).

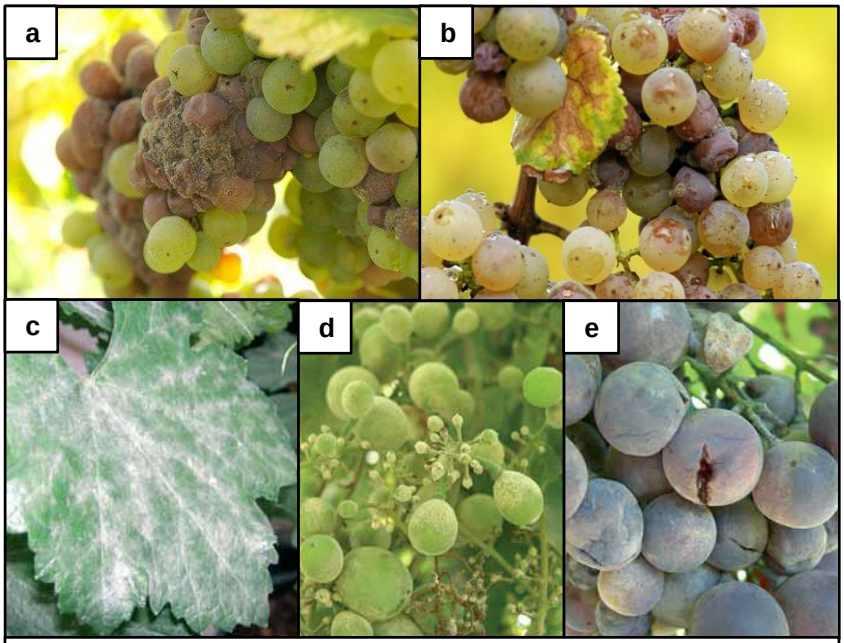

Figure 1.2. Grapevine Gray mold (a,b) and Powdery mildew (c, d, e). a, b: "noble rot" on grapes; c, d and e: different symptoms of *E. necator* on leaf and bunches.
Pictures a and b from Maack T., Rheingau, Germany, October 2005. Pictures c, d and e from the "Archive SafeCrop Centre", Edmund Mach Foundation (FEM).

1.1.3.3. Black rot

Grapevine black rot is caused by the ascomycete *Guignardia biwellii*, which attacks grapevine during hot and humid weather. Primary infections affect generally young leaves and fruit pedicels, appearing as tiny red necrotic spots which may cover the whole leaf surface and that subsequently enlarge (Fig. 1.3a). As the disease progress, the spots turn into brown and their margins become black. On the upper side of the spot picnidia are rapidly formed and their dispersal through wind and rainfalls favours secondary infections, that may develop also on berries and stems. On berries, black rot forms brown areas surrounded by

a black line margin with a flat or depressed central region. Picnidia are initially produced in the spot centre and, if not controlled, they number increase until the berry shrinks and becomes rotten (Fig. 1.3b) (Agrios 1997).

1.1.3.4. Eutypa dieback

Eutypa dieback is a wood decay disease caused by the ascomycete *Eutypa lata*. Symptoms do not usually appear until grapevines are at least six years old. Shoot symptoms are most evident during the beginning of the spring, with shoot arising from infected trunks being stunted with small chlorotic leaves (Fig. 1.3c-d) (Moller and Kasimatis 1978). Berries fail to develop or develop very poorly, inducing yield losses ranging from 30 to 60% on highly susceptible cultivars (Munkvold and Marois 1994). *Eutypa* dieback shoot symptoms are always accompanied by a canker, which often appears V-shaped in a cross-section of the perennial wood (Fig. 1.3c). Cankers progress toward the trunk, killing the distal portions of the grapevine, and eventually, the entire grapevine may die in an average period of 10 years after the initial infection (Pascoe, 1999, Gomès and Thévenot, 2009). Currently, there is no cure for *Eutypa* dieback.

1.1.3.5. Esca

Esca – also known such as “apoplexy” or “lack measles” – is a complex trunk disease involving at least five fungi: *Fomitiporia punctata*, *Stereum hirsutum*, *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora*, and *E. lata*, which obstruct the vascular system (Larignon and Dubos 1997). It affects both young and older grapevines. Cross-section in infected trunks shows a central soft, white necrosis (touchwood), surrounded by a brownish hard zone. Typical symptoms on leaves consist of chlorotic, rounded or irregular spots between the veins or along the leaf margins (Fig. 1.3e) while dark, tiny spotting of the grapes is called “black measles” (Fig. 1.3f) (Mugnai et al. 1999). Esca develops slowly in the grapevine until the plant exhibits a sudden apoplectic decline, eventually killing the grapevine within a few days (Gomès and Thévenot, 2009). No chemical is currently available to control Esca disease.

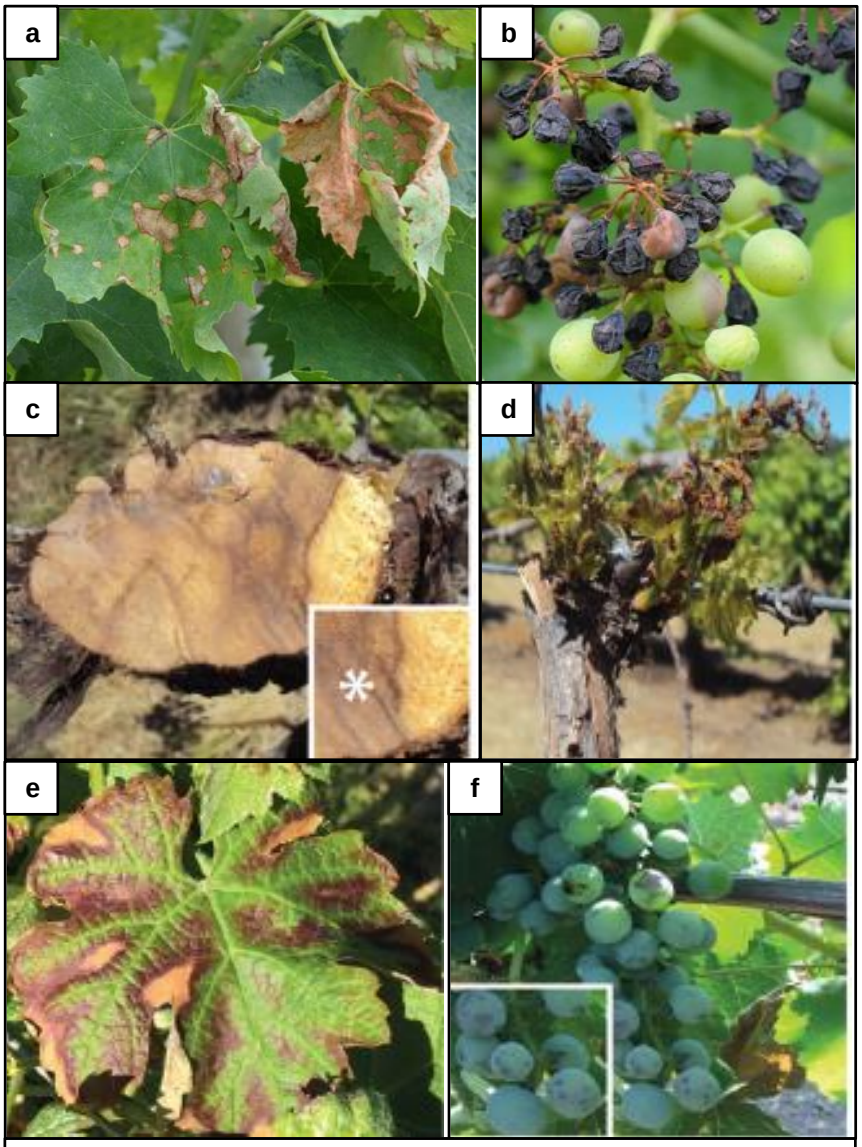

Figure 1.3. Grapevine Black rot (a, b) and wood decay disease (c-f). a and b: different symptoms of *Guignardia biwellii* on leaves and clusters. c and d: different symptoms of *Eutypa lata* on wood and shoots. e and f: typical foliar symptoms and berry spotting (measles) associated with *Esca*.
Pictures a and b from Blancard D., INRA 2013; pictures c, d, e and f from Morales-Cruz et al. 2015.

1.1.3.6. Crown gall disease

Grapevine crown gall disease is caused by the bacterium *Agrobacterium vitis*; it can be defined as a biotrophic pathogen since it maintains a parasitic relationship with living tissues of the host to complete its lifecycle. Virulent strains of this bacterium induce the formation of tumorigenic structures at the site of infection for nutrient uptake (Fig. 1.4a), while necrosis and a HR-like response has been reported in grapevine roots and in non-

host plants infected with this bacterium, respectively. The infection starts commonly through plant injuries, particularly by freezing and/or mechanical wounds, to favor the release of phenolic compounds that act as chemoattractants. These compounds activate the transcription of bacteria *vir* genes, whose products induce the transfer of the T-DNA into the host genome (Armijo et al. 2016). T-DNA sequences are involved in opine synthesis – afterwards catabolized by *A.* strains – and gall induction (Escobar and Dandekar, 2003).

1.1.3.7. Pierce's disease

Pierce's disease is caused by the gram-negative, xylem-limited bacterium *Xylella fastidiosa*. It is a disease that affects wine, table and raisin grape production. The symptoms of this disease can be severe, including leaf scorching (Fig. 1.4b), desiccated fruit, cordon die back, and finally grapevine death (Roper et al. 2007). This bacterium can be classified as a biotrophic pathogen, because it does not kill the host tissue until later stages of its life cycle. The optimum growth conditions are warmer environments close to 28˚C (Lieth et al. 2011). *X. fastidiosa* is transmitted to new host plants by insect vectors, infects the grapevine creating a biofilm in xylem vessels that disrupts water and nutrients flow throughout it. This occlusion is composed by host gums, bacterial exopolysaccharide or degradation products from the host cell wall (Armijo et al. 2016).

1.1.3.8. Fanleaf virus (GFLV)

To date, 70 virus species able to infect the *Vitis* genus have been identified, causing at least 25 different diseases in grapevine (Martelli, 2014); one of the most representative species is the grapevine fanleaf virus (GFLV). This virus is transmitted by the dagger nematode *Xiphinema index*. It causes typical fan-leaf degeneration, leaf decline (Fig. 1.4c-d) and reduced fruit quality. It causes abnormal bifurcations and deformations in the development of internodes on canes, which may result in the appearance of double or shortened internodes. In addition, GFLV is considered the major threat to grape industry due to its ability to reduce crop yield up to 80% depending on the isolate, the susceptibility of the grapevine variety and environmental factors (Martelli, 2014).

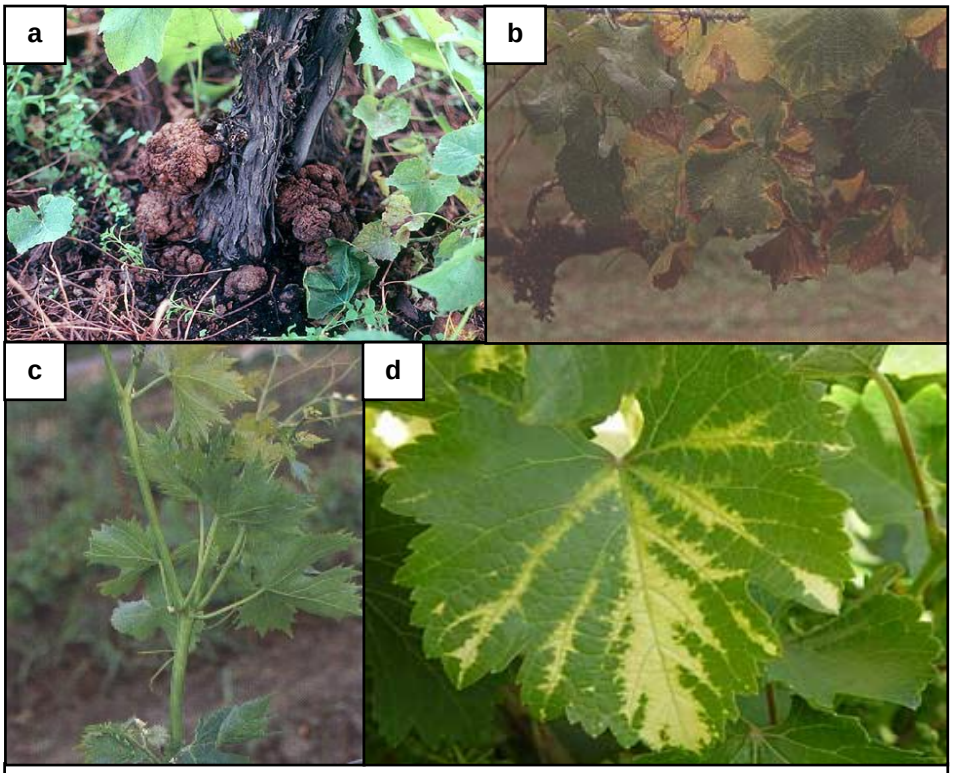

Figure 1.4. Grapevine crown gall disease (a), Pierce's Disease (b) and fanleaf virus (c-d). a: tumorigenic structures on trunk caused by *Agrobacterium vitis*. b: leaf scorching caused by *Xylella fastidiosa*. c and d: leaf symptoms caused by fanleaf virus (GFLV).
Pictures a and d from Canadian Food Inspection Agency, pictures b and c from www.fao.org.

1.2. GRAPEVINE DOWNY MILDEW

1.2.1. Grapevine downy mildew life cycle

DM is caused by the obligate biotrophic oomycete *P. viticola* (Berk. & M.A. Curtis) Berl. & De Toni, belonging to the order *Peronosporales*. *P. viticola* is a diploid, heterotallic pathogen (Wong et al. 2001) with asexual multiplication cycles over the period of grapevine vegetative growth and a sexual overwintering phase (Fig. 1.5). The former leads to production of spores for secondary infections, the latter to quiescent oospores for primary infections (Deacon J, 2006). It overwinters as oospores embedded in fallen leaves and other host tissues infected in the previous season. In spring, with temperature above 10°C and rain precipitation, oospores germinate and form an elongated germ tube terminating in a macrosporangium. The oospore nuclei migrate in this structure and – through mitotic divisions – the zoospores are formed. Afterwards, the zoospores are released and dispersed onto grapevine tissues by rain or wind (Spencer, 1981), but also relying on oospore human transfer for longer distances dispersal (Gobbin et al. 2003a). Zoospores swim in free water on the grapevine surface towards a stoma, where they lose flagella, encyst and secrete a glicoproteic extracellular matrice to attach to plant-surface (Grenville-Briggs and Van West 2005). Zoospores then produce the appressorium and the germinative tube through the reallocation and synthesis of actin and tubulin (Riemann et al. 2002). The germinative tube penetrates through the stoma inside the host tissue and develops a substomatal vesicle. A primary hypha emerges from the vesicle, grows in the mesophyll, branches at intervals in specialized intracellular structures – named haustoria –, and gives rise to the intercellular mycelium (Unger et al. 2007). After a variable incubation time, sporulation take places during warm and humid nights. Sporangiophores, bearing sporangia, emerge through stomata. The sporangia, where zoospores are formed, detach and are dispersed by wind and rain splash to new host tissues, marking the start of a new infection cycle. These secondary cycles of infection, under favourable weather conditions, can occur repeatedly throughout the grapevine growing season (Gobbin et al. 2003a, Gessler et al. 2011) Towards the end of the growing season sexual reproduction occurs within the infected host tissue through the fertilisation of oogonia by antheridia (Wong et al. 2001, Deacon J, 2006). The resulting sexual spore is an oospore, which is the survival stage of the pathogen representing the primary inoculum for the next season, as well as a source of genetic variation (Deacon J,

2006). The organism is diploid in both sexual and asexual stages (Rumbou and Gessler, 2003).

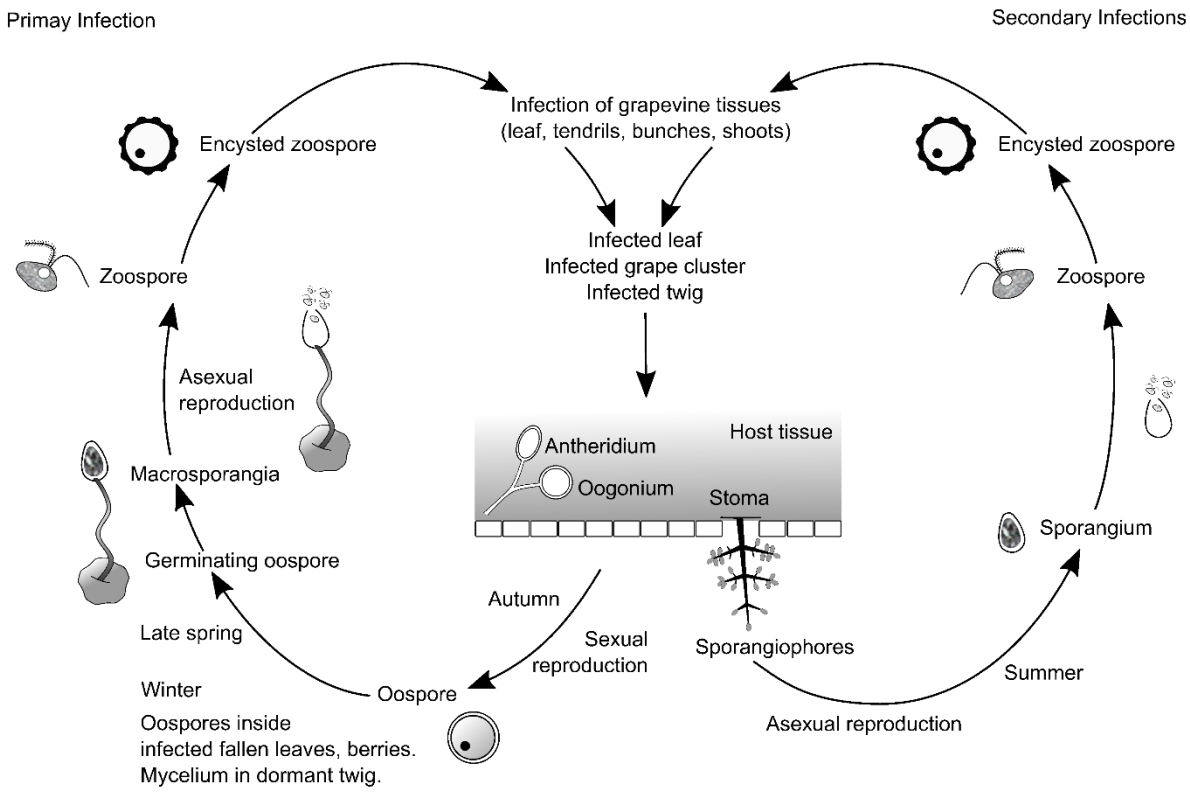

Figure 1.5. Disease cycle of *P. viticola*, the causal agent of grapevine downy mildew (Berk. & M.A. Curtis) Berl. & De Toni. (from Buonassisi et al., submitted)

1.2.2. Grapevine downy mildew symptoms

P. viticola infects all green parts of the host plant through the stomata. It generally causes yellow discoloration, necrosis and distortion. On young leaves, lesions appear as yellow, translucent “oil spots” with a chocolate-brown halo (Fig. 1.6a). Multiple oil-spots can coalesce to cover much of the leaf surface. Oil spots become dry and necrotic as they age, first in the centre and later throughout the entire lesion. Sporulation only occurs on the abaxial leaf surface, where the stomata reside (Fig. 1.6b); suited conditions for sporulation are saturating humidity (>93%) and temperatures of 18-20°C. The sporangiophores and sporangia appear as a white, downy, cottony growth. Under highly favourable conditions, sporulation may appear on the abaxial leaf surface before the yellow oil spot becomes visible

on the adaxial leaf surface. On older oil spots, sporulation occurs primarily on the margins of the lesion. On older leaves, the lesions are restricted by veins to form small, angular, yellow to reddish-brown spots, which combine to form a patchwork or mosaic-like pattern, especially towards autumn season (Fig. 1.6c) (Lafon and Clerjeau, 1988).

Infected inflorescences and young berries appear yellow or grey and may be covered with cottony spores (Fig. 1.6d). Infected shoot tips and rachises of young inflorescences distort into a curl or corkscrew (Fig. 1.6e-f), and the sporulation can occur also on pedicels and berries (Fig. 1.6g-h). Clusters infected at an early stage can result in individual berries, sections of the cluster, or even entire clusters turning brown, drying and falling off the grapes. Berries infected later in the season (after 2-3 weeks post-bloom) become discoloured and shrivel but do not support sporulation; this stage is sometimes referred to as the “brown rot” phase (Fig. 1.6i) (Lafon and Clerjeau, 1988).

Generally, resistant genotypes are characterized by necrotic points or flecks and little or absent sporulation on the leaf borders. The presence of necrotic tissue restricted to infection sites, indicates ongoing hypersensitive reaction (HR), which is a programmed cellular suicide triggered also by pathogen attack (Beers and McDowell 2001). HR is considered one of the most common and effective plant weapons to hamper biotrophic pathogen growth, though it facilitates infections by necrotrophics at the same time (Govrin and Levine, 2000)

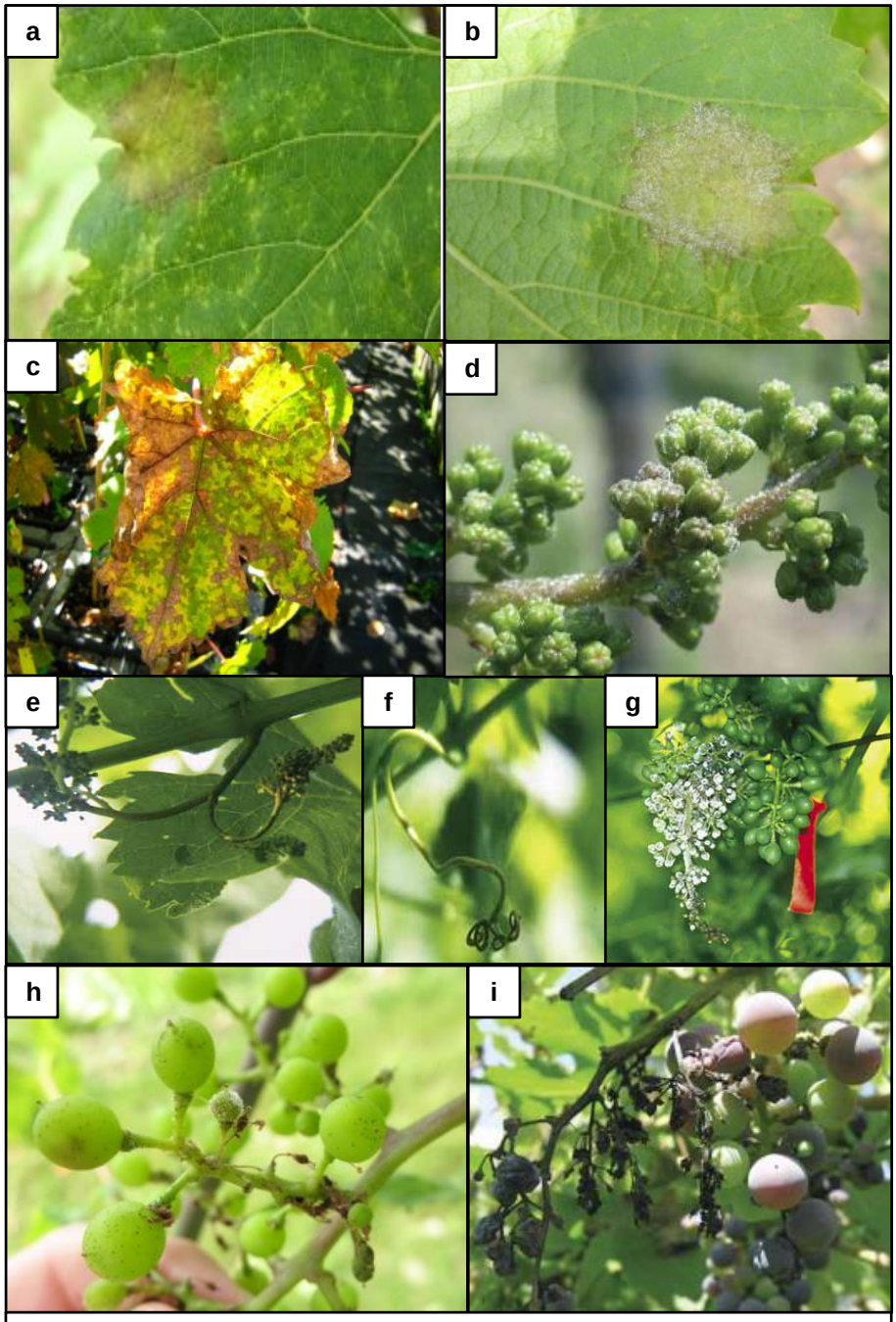

Figure 1.6. *Plasmopara viticola* disease symptoms. a: “oil spot” on adaxial leaf surface; b: whitish gray mold on abaxial leaf surface; c: mosaic-like pattern on older leaves; d: gray mold on young inflorescence; e and f: curl or corkscrew structure on young inflorescence (e) and tendril (f); g and h: gray mildew form on unripe clusters; i: “brown rot” on ripe grapes.
Pictures a,b, c and h from Peressotti, 2009. Pictures d, e, f, g and I from the “Archive SafeCrop Centre”, Edmund Mach Foundation (FEM).

1.2.3. Grapevine downy mildew disease control

Depending on the environmental conditions, numerous clonal infection cycles may occur in one season seriously affecting the final grape yield and quality. Therefore, wine and table grape production is guaranteed by the massive use of fungicides. There are multiple pre- and post-infection chemicals available such as copper- and sulphur-based compounds. First applications are generally advised at 3-8 inches of shoot-growth, immediate pre-bloom, and post-bloom to protect the young inflorescences and fruit. For the remainder of the season, sprays may be based on a routine schedule (usually every 10-14 days) to maintain continuous protection of the vines. Alternatively, sprays may be based on disease risk as determined by weather conditions and forecasting models. Most computer-based forecasting models for DM management incorporate temperature, rainfall, relative humidity and leaf wetness. Simulators more complex incorporate information on host growth stage and varietal susceptibility (Lafon and Clerjeau, 1988, Viret et al., 2001). DM management must be rigorous in countries with temperate-humid climate conditions, such as eastern North America and parts of Europe, and during unusually wet seasons in dry locations such as California or Australia. It has been estimated that the European Union employs 68.000 tons/year of fungicides to control grape diseases, equalling 65% of all fungicides used in agriculture, but only 3.3% of the EU arable soils are occupied by grapevine (Eurostat report 2007).

In lack of treatments and with favourable weather conditions DM can devastate up to 75% of the crop in one season and weaken newly born shoots, causing a serious economic loss. Nevertheless, the repeated and massive use of fungicides leads to environmental pollution, development of resistance and residual toxicity and it fosters human health concerns (Pimentel, 2005). Some estimations indicate that less of 0.1% of fungicides applied to crops actually reach the intended pathogen. The remainder accumulates in soils, where it may filter into ground or surface water and prove toxic to microorganisms, aquatic animals and humans. Exposure of birds and bees to pesticides can cause reproductive failure, or even kill them directly in high enough doses. Domesticated livestock may also be affected by exposure to pesticides (Wilson et Tisdell, 2001).

An alternative approach in DM management, with the potential to greatly reduce the pesticide application and thereby leading to a substantial contribution to viticulture sustainability, is represented by taking advantage of innate disease resistance/tolerance present in several wild American and Asian *Vitis* species.

1.2.4. Grapevine breeding for disease control

Targeted breeding activities started around the beginning of the nineteenth century, predominantly in North America. The colonists failed to grow the delicately flavoured *V. vinifera* vines, due to severe frost damage as well as the destruction of the grapes by pests or fungal diseases. On the other hand, the sturdy native American grapes produced strong-flowered wines that they did not like. During the following decades, breeders selected a plethora of cultivars so-called “American hybrids”, among which Diamond. In Europe, resistant breeding was introduced in the second part of nineteenth century due to the introduction of *Phylloxera vitifoliae* and DM. Many private French breeders – so-called “direct producers” – started their own breeding programs creating thousands of new cultivars, but many of these failed. (reviewed in Eibach et Topfer, 2015). The so-called “French hybrids”, represent a highly valuable genetic resource carrying a combination of resistance and quality. Indeed, several breakthroughs have been achieved in grapevine resistance breeding during the 20th century when over 6,000 hybrids were registered in Europe. Unfortunately, most of these varieties did not succeed in the market due to poor wine quality or other agronomical factors (Pacifico et al. 2013). However in the last decades, newly bred wine grapevine cultivars, displaying field disease resistance and high wine quality, have been introduced in the market (Guedes de Pinho and Bertrand 1995), such as the Hungarian grapevine hybrid Bianca or the German Regent. Recently, many fungus-resistant grapevine varieties, presenting advantageous agronomic attributes and enological characteristics, have been developed in North America (named FRG) and Europe (named PIWI) for conventional and especially organic farming (Pedneault and Provost 2016).

1.3. PLANT-PATHOGEN INTERACTION

Plants, unlike mammals, lack mobile defender cells and a somatic adaptive immune system. Instead, they rely on the innate immunity of each cell and on systemic signals emanating from infection sites. Indeed, to prevent diseases caused by pathogens, plants rely on a sophisticated defence mechanisms, able to detect the presence of potential pathogens and to activate a suitable response (reviewed in Chisholm et al. 2006, Jones and Dangl 2006, Dodds and Rathjen 2010, College and Williams, 2012; Fawke et al. 2015).

1.3.1. The plant immune system

To defend themselves against the different types of pathogens, plants have an array of structural barriers and preformed antimicrobial metabolites (Pieterse et al. 2009). Regardless from pathogen attack, so-called “constitutive defences” are generally referred to morpho-anatomical characteristics of grapevine organs such as leaf hairs, stomata and cuticular membrane (Muganu and Paolocci 2013). Among the “constitutive compounds”, the phytoanticipins are the most important and they are present before challenge by microorganisms or are produced after infection solely from preexisting constituents (VanEtten et al. 1994). Many pathogens succeed in breaking through this pre-invasive layer of defense. However, a broad spectrum of inducible plant defences can be recruited to limit further pathogen ingress.

The first defence level, named Pathogen Associated Molecular Patterns (PAMP)-Triggered Immunity (PTI), is a basal immune response activated by the recognition of conserved molecules (or patterns). Specific Pattern Recognition Receptors (PRRs), localized on the plasma membrane of plant cells, perceive the presence within the apoplast of pathogen and host-derived, released during infection (reviewed in Newman et al. 2013, Raaymakers and Ackerveken 2016). PAMPs are molecular patterns, usually exposed, present in molecules essential to the pathogen, and therefore highly conserved (Fig. 1.7a). Plant-derived patterns, also known as damage-associated molecular patterns (DAMPs), are released from the host as a result of the infection process. Damage perception and pathogen perception therefore act together in promoting PTI. Normally this first response is sufficient to defeat the invading microorganism, however some of them have become able to counteract PTI through the secretion of specific proteins called effectors, which can suppress the plant immune response and manipulate the physiology of the host cell to the

pathogen benefit, e.g. promoting nutrient leakage. (reviewed in Bozkurt et al., 2012). This phase is called effector-triggered susceptibility (ETS) (Fig. 1.7b). Successful pathogens are able to produce a broad range of effectors, extremely variable in structure and function, in a species- or race-specific way. Effectors can be apoplastic, functioning into the plant extracellular space, or cytoplasmic, translocated inside the plant cell (Kamoun 2006, Bozkurt et al. 2012)

In turn plants evolved a second defence level, effector-triggered immunity (ETI), which relies on a second class of receptors, the resistance proteins (R), that recognize in a highly specific way the effectors presence and/or activity (Fig. 1.7c) (reviewed in Cui et al., 2015). The majority of these R proteins are intracellular receptor proteins of the nucleotide-binding leucine-rich repeat (NB-LRR) type (Cui et al. 2015). The recognition between effectors and R proteins results in a strong selective pressure for both the pathogen and the host plant. The pathogen endlessly tries to avoid ETI by diversifying its own effectors or by acquiring new ones. The plant in turn evolves new receptors so that ETI is triggered again. This coevolution, characterized by reiterating cycles of selection of new effectors and receptors, has been described as “zigzag” model by Jones and Dangl (2006). Whereas PPRs and PAMPs tend to be widely conserved, effectors and receptors are codified by non-essential genes and thus can rapidly evolve (Dodds and Rathjen 2010). PTI and ETI defence mechanisms are similar, although ETI is generally considered faster, stronger and active for longer periods. Downstream of PTI and ETI signaling events, plants respond by activating a large number of integrated defence responses to arrest the pathogen. These defences include cell wall fortification through the synthesis of callose and lignin; the production of antimicrobial secondary metabolites, such as phytoalexins; and the accumulation of pathogenesis-related (PR) proteins, such as chitinases and glucanases, that degrade fungal and oomycete cell walls (Pieterse et al. 2009). The activation of ETI leads to disease resistance and is often associated to a cell death response localized at the infection site to prevent pathogen nutrition and growth. This reaction is called hypersensitive response (HR), characterised by the massive production and accumulation of reactive oxygen species (ROS), among which hydrogen peroxide (H_2O_2).

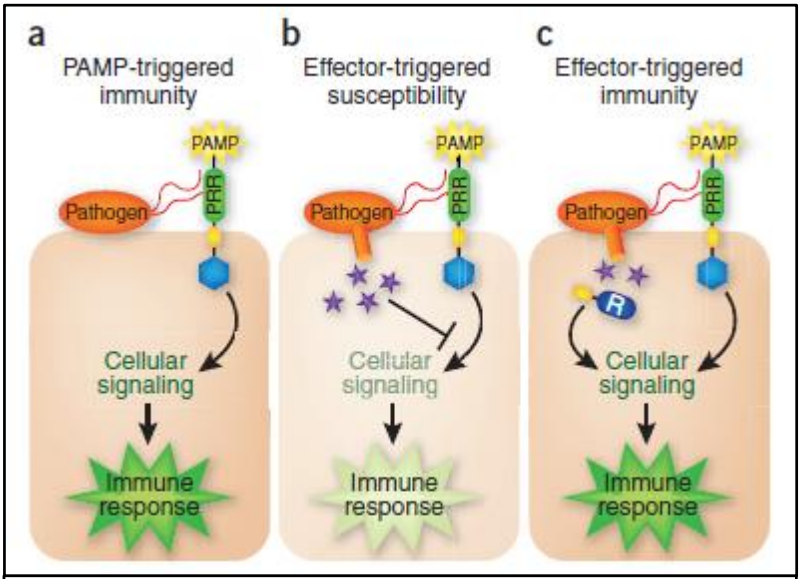

Figure 1.7. Simplified schematic representation of the plant immune system. (a) Pathogen-associated molecular patterns (PAMPs)-Triggered Immunity (PTI); b) Pathogen effectors (purple stars) that suppress PTI, resulting in effector-triggered susceptibility (ETS). (c) Resistance (R) proteins that recognize the effectors, resulting in a secondary immune response called effector triggered immunity (ETI). (from Pieterse et al. 2009)

1.3.2. Hormone signalling

Pathogen infection stimulates the plant to synthesize one or more hormonal signals depending on the type of attacker and regulating the defence network (De Vos et al. 2005). Biotrophic pathogens are generally sensitive to defence responses that are regulated by salicylic acid (SA), whereas defences that are controlled by jasmonic acids (JAs) and ethylene (ET) commonly deter pathogens with a necrotrophic lifestyle (Glazebrook 2005). The wound response and against insect herbivores is regulated by the JA signaling pathway as well (Howe, G.A. 2004, Pieterse et al. 2009).

1.3.2.1. Systemic immunity

Once plant defence responses are activated at the site of infection, a systemic defence response is often triggered in distal plant parts to protect these undamaged tissues against subsequent invasion by the pathogen. This long-lasting and broad-spectrum induced disease resistance is referred to as systemic acquired resistance (SAR) (Durrant

and Dong, 2004) and is characterized by the coordinate activation of a specific set of PR genes, many of which encode for proteins with antimicrobial activity (Van Loon et al., 2006). The onset of SAR can be triggered by PTI- and ETI-mediated pathogen recognition and is associated with increased levels of SA, locally at the site of infection and or in distant tissues (De Wit, 1997, Mishina and Zeier, 2007, Pieterse et al. 2009)

Beneficial soil-borne microorganisms, such as mycorrhizal fungi and plant growth–promoting rhizobacteria, can induce a phenotypically similar form of systemic immunity called induced systemic resistance (ISR) (Van Loon, et al.,1998, Pozo et al. 2008). Like PAMPs of microbial pathogens, different beneficial microbe-associated molecular patterns are recognized by the plant, which results in a mild but effective activation of the immune response in systemic tissues (Van Wees et al.,2008, Bakker et al. 2007, Van der Ent et al. 2008). In contrast to SA-dependent SAR, ISR triggered by beneficial microorganisms is often regulated by JA- and ET-dependent signaling pathways and is associated with priming for enhanced defence rather than direct activation of defence (Van Wees et al., 2008, Conrath et al. 2009, Pozo et al. 2008, Pieterse et al. 2009).

1.4. "OMICS" APPROACHES TO STUDY THE GRAPEVINE-DOWNY MILDEW INTERACTION

The development of controlled infection protocol and contemporary "omics" approaches (next generation sequencing/genomics, QTLomics, transcriptomics, proteomics and metabolomics) associated to comparative studies are shedding light into the early host responses to DM attack and into the complex plant defence mechanisms. In this section are presented the main "omics" approaches used to study grapevine-DM interaction.

1.4.1. Phenomics

In viticulture, DM represents a relevant case of dual epidemics. Dual epidemics are infections developing on two or several plant organs during the same cropping season. Agricultural pathosystems where they occur are often considerable, because the harvestable and valuable part is one of the organs affected (Savary et al. 2009). Grapevine dual epidemics are often difficult to manage, because prediction of the risk toward the harvestable grapes has been scarcely and only recently investigated (Savary et al. 2009, Calonnec et al. 2013, Vezzulli et al. submitted). In the symptom assessment procedure a crucial role is played by the ontogenic resistance, also termed age-related resistance, occurring in various organs. In particular, the fourth and fifth fully expanded leaves do not show ontogenic resistance yet, whereas the older leaves of the bottom of the grapevine shoots present a higher resistance to DM. Analogously, the grapevine phenological stage is of paramount importance for the DM response evaluation also on inflorescence/clusters. This is linked to the presence/absence of functional stomata (Nakagawa et al. 1980, Reuveni et al. 1998, Kennelly et al. 2005, Steimetz et al. 2012).

P. viticola symptoms can be assessed by different methods: i) macroscopic and microscopic inspection, ii) image-based, staining and ultrastructural analyses, and iii) metabolomic profiling.

1.4.1.1. Macroscopic and microscopic inspection

Traditionally, evaluation of DM symptoms occur in untreated field upon natural infection. Vineyard evaluation is a relevant method for directly rating DM resistance because breeders want to ascertain resistance in field conditions (Kono et al. 2015). Most visual

observations are focused on the resistance and/or susceptibility to DM infections, at both foliage and cluster level, in different genetic backgrounds, from *Vitis* species and hybrids, through *V. vinifera* varieties, to clones (e.g. Demaree, 1937; Eibach et al. 1989, Basler and Pfenninger 2003, Cindric et al. 2003, Pavloušek 2012, Wan et al. 2007, Boso et al. 2011, Pacifico et al. 2013). Moreover, several studies have been performed comparing foliar assessments under field and laboratory conditions (e.g. Brown et al. 1999, Cadle-Davidson 2008, Prajongjai et al. 2014).

In the last two decades, it has been paid attention also to DM inoculation experiments in controlled conditions. These procedures are highly reproducible, supporting genetics and other disciplines that need to be independent from the environmental/field effect. As regards plants grown in greenhouse, *in vivo* artificial inoculations with DM spores have been performed mainly on woody cuttings to evaluate leaf response (e.g. Perazzolli et al. 2011, Boso et al. 2008, Gindro et al. 2006), meanwhile the other organs response is poorly studied. Another possibility to reproduce DM natural infections on entire plants consists of *ex vivo* artificial inoculations on regenerated individuals grown in growth chamber (Kortekamp and Zyprian 2003 and Deglène-Benbrahim et al. 2010). Most of the *in vitro* tests developed so far for DM symptom assessment are based on leaf disc bioassays: this procedure has the great advantage to standardize the phenotyping procedures and annotations (e.g. Staudt and Kassemeyer 1995, Boso and Kassemeyer 2008, Jürges et al. 2009, Peressotti et al. 2010, Toffolatti et al. 2012). Indeed bioassays performed on tissues still attached to entire plants might be affected by non-homogeneous environmental conditions (e.g. light exposure in greenhouse) (Boso and Kassemeyer 2008, Kiefer et al. 2002, Cadle-Davidson 2008). Leaf disc bioassay results are robust predictors of field resistance/susceptibility at leaf level (e.g. Boso et al. 2014, Brown et al. 1999, Sotolář 2007), but little is known on other organs such as the inflorescences.

All the experiments mentioned above have been performed based on visual/macroscopic and microscopic observations carried out using light microscopes and stereomicroscopes. To standardize the DM symptom assessment, annotations were executed mainly referring to three international codes supported by the European and Mediterranean Plant Protection Organization (OEPP/EPPO, 2001), the *Organisation Internationale de la Vigne et du Vin* (OIV, 2009), and the International Union for the Protection of New Varieties of Plants (UPOV, www.upov.int). EPPO code is based on two parameters: disease severity (DS; expressed as a percentage of the organ area showing symptoms of sporulation) and disease incidence (DI; calculated as the number of organs

with sporulation/total number of organs). By contrast, OIV and UPOV codes both refer to a discrete scale, but differently: the first rating system is based on five classes (1, 3, 5, 7 and 9) ranging from the most susceptible (1, extended sporulation) to the totally resistant (9, no symptoms at all) genotype, while the second code is based on the same classes but with opposite meaning. An additional parameter that might be considered for DM resistance evaluation is the necrosis presence, although it is not considered a robust index since it can be associated to host and non-host resistance and could be affected by various physiological factors (Bashir et al. 2013, Heath 2000). Along with the DS parameter, the OIV descriptors are the most widely deployed. In particular, OIV 452 and OIV 453 are related to in field and *in/ex vivo* DM symptom evaluation on leaves and inflorescence/clusters, while OIV 452-1 refers to *in vitro* DM response assessment on leaf discs. To date, no OIV descriptors for *in vitro* DM symptom assessment on other organs is available.

1.4.1.2. Image-based, staining and ultrastructural analyses

To increase accuracy and improve precision of disease symptom assessment, image analyses were carried out using commercial software. At first, the leaf area occupied by DM spot on the not picked leaves was calculated using digital photographs and the analySIS 3.0 software (Boso et al. 2004). Subsequently, to quantify the leaf disc area infected by the pathogen, an image analysis based on a semi-automatic and non-destructive method was developed, using the open source software ImageJ (Peressotti et al. 2011). Recently, Khiook et al. 2013 used ImageJ and Visilog 6.9 software to detect the sporulation on leaf disc surface infected by *P. viticola*, the leaf area colonized by the pathogen after aniline blue staining, and the percentage of the leaf area showing H_2O_2 accumulation. Moreover, staining analyses have been carried out to reveal the pathogen presence. The aniline blue fluorescence was the main method used for epifluorescence microscopy. *P. viticola* appeared as blue structures in the infected leaves (Hood and Shew, 1996, Unger et al. 2007, Díez-Navajas et al. 2006, Díez-Navajas et al. 2008, Boso et al. 2010), meanwhile the inflorescences, flower clusters and rachis are poorly investigated. Moreover, histochemical visualization was undertaken using the DAB staining technique based on polymerization of diaminobenzidine in presence of H2O2 and characterized by a reddish-brown coloration (Thordal-Christensen et al., 1997). Indeed, in the resistant genotypes H_2O_2 production is one of the defence mechanisms activated in the site of leaf infection (Trouvelot et al. 2008, Liu et al., 2014). Finally, transmission electron microscopy was used to observe cellular

interactions between *P. viticola* and grapevine leaf tissues (Musetti et al. 2005, Musetti et al. 2006).

1.4.1.3. Metabolic profiling

Metabolic profiling of host-pathogen (DM) interaction have been performed by measuring grapevine metabolites with various analytical instruments. The most studied compound class is represented by phenilpropanoids. δ-viniferin, a resveratrol dehydrodimer, produced *in vitro* by the oxidative dimerization of resveratrol by plant peroxidases or fungal laccases, was identified in wines and in grape cell cultures (Langcake and Pryce 1977). This compound was also detected by nuclear magnetic resonance (NMR), high-performance liquid chromatography-diode array detection (HPLC-DAD), and HPLC-mass spectrometry (MS) in grapevine leaves infected by DM. To rapidly evaluate the level of DM resistance at different time-points, the metabolic changes occurring during the interaction of *P. viticola* with two different cultivars – Regent (resistant) and Trincadeira (susceptible) – were investigated by NMR spectroscopy. Metabolites responsible for their discrimination were identified as a fertaric acid, caftaric acid, quercetin-3-O-glucoside, linolenic acid, and alanine having higher levels in the resistant hybrid, while the susceptible cultivar showed higher levels of glutamate, succinate, ascorbate and glucose (Ali et al. 2012). Furthermore, several biomarkers for the prediction of DM resistance and susceptibility at leaf level were identified. In particular, 16 individual metabolites – g-tocopherol, squalene, a-amyrine, stigmasta-3,5-diene-7-one, hexahydrofarnesyl acetone, glycolic acid, 3-hydroxybutanoic acid, 3-hydroxycaproic acid, malic acid, tartaric acid, erythronic acid, arabinoic acid, monoethyl phosphate, undecyl laurate and isopropyl myristate – were proposed as biomarkers for DM, as well as powdery mildew and botrytis foliar resistance (Batovska et al. 2008). To better understand the host–pathogen relationships, Becker et al. (2013) have recently evaluated also the direct flow injection by electrospray – Fourier transform ion cyclotron resonance MS of leaf extracts as a rapid method for the study of grapevine response to DM attack. The comparison of MS profiles obtained from control and infected leaves of different levels of resistant grapevines highlights several classes of metabolites (mainly saccharides, acyl lipids, hydroxycinnamic acids derivatives and flavonoids). Statistical analyses of 19 biomarkers showed a clear segregation between inoculated and healthy samples. In particular, relative high levels of disaccharides, acyl lipids and glycerophosphoinositol were

detected in inoculated samples; sulfoquinovosyl diacylglycerols also emerge as possible metabolites involved in plant defence.

1.4.1.4. Genetics coupled with Genomics

1.4.1.4.1. Grapevine

To access the genetic variation required for grapevine breeding, the first step is the characterization of genetic resources, typically stored as *ex situ* collections. In the last two decades, a big attempt has been performed by the international grapevine research community that defined reference microsatellite (SSR) markers and analysis protocols to identify the true-to-type genotype (This et al. 2004, Maul et al. 2012) (Table 1.1). Molecular markers - microsatellites and single nucleotide polymorphisms (SNPs) - have also clarified the structure of genetic diversity making more accessible the vast germplasm collections for grapevine breeding programs (Bacilieri et al. 2013, Emanuelli et al. 2013). In addition, core-collections which maximize the genetic diversity can be employed in *ad hoc* studies (e.g. Le Cunff et al. 2008; Nicolas et al. 2016).

From the first grapevine genetic map (Lodhi et al. 1995) a long way has been undertaken: out of the numerous genetic (linkage) maps published, a consensus (Adam-Blondon et al. 2004) and an integrated (Vezzulli et al. 2008) ones were chosen as a reference for chromosome/linkage group (LG) number and marker distribution. Concerning physical maps, whole genome ones were constructed for *V. vinifera* cv. Cabernet Sauvignon (Moroldo et al. 2008) and cv. Pinot Noir (Scalabrin et al. 2010), and represent a useful and reliable intermediary tool between a genetic map and the genome sequence. These research works culminated into the publication of two grapevine genome sequences: the near-homozygous line PN40024 (Jaillon et al. 2007) sequenced with the Sanger technology – thus chosen as the species reference genome, and the highly heterozygous Pinot Noir ENTAV115 clone (Velasco et al. 2007) resolved through Sanger shotgun sequencing and highly efficient sequencing by synthesis (SBS). Recently, a next-generation sequencing technology named Single Molecule Real-Time, along with associated bioinformatics tools, made possible the assembly of the phased Cabernet Sauvignon genome (Chin et al. 2016). Re-sequencing plays a pivotal role in deciphering the grapevine genome information, allowing the identification of an unlimited number of markers as well as the analysis of

germplasm allelic diversity based on allele mining approaches (Barabaschi et al. 2016). Several re-sequencing projects are now ongoing not only on *V. vinifera* cultivars, but also on *Vitis* hybrids. The complete determination of the *V. vinifera* genome sequence has led to the identification of putative resistance genes and defence signalling elements (Casagrande et al. 2011) as well as of nucleotide-binding site - resistant (*NBS-R*) gene clusters (Malacarne et al. 2012). With the introduction of deep sequencing, a complex gene family encoding nucleotide-binding leucine-rich repeat (NBS-LRR) proteins was identified, whose members have been isolated and characterized (Seehalak et al. 2011). However, full functional characterization of *Vitis* resistance gene analogs (RGAs) has not been widely reported (Fan et al. 2015). Under this perspective, the genomic sequence information of non-*vinifera* genotypes is of paramount importance because they carry several agronomic traits foreign to most *vinifera* varieties; these characteristics, which derived from the ancestral *Vitis* species, encompass resistance to biotic stresses, DM included.

To dissect the genetic bases of DM resistance, various genotype-phenotype association approaches have been employed, from the traditional Quantitative Trait Loci (QTL) analysis (Table 1.2) till the studies based on pedigree information and inference (Di Gaspero et al. 2012, Peressotti et al. 2015). Genome Wide Association Studies (GWAS) and the last frontier of Genomic Selection (GS) are alternative strategies, borrowed from livestock breeding, which allow to study characteristics of interest with a polygenic base, such as grape and wine quality-related traits (reviewed in Di Gaspero and Foria 2015). Given the recent progress in genomics and DM phenomics, a more accurate and comprehensive characterization of the QTLs associated to DM resistance is possible. As proposed for other crops, it is appropriate to extend the “QTLome” concept also to grapevine. The QTLome of a specific trait is defined as the set of information describing all the experimentally supported QTLs for the desired characteristic in one species (Salvi and Tuberosa 2016). Nowadays, the amount of DM resistance QTLome information is vast, but how to convey this know-how to breeders and end-users needs to be improved.

Genetics is now irreversibly coupled with genomics and together they have bolstered Marker-Assisted Selection (MAS) and Marker-Assisted Breeding (MAB) also in grapevine, a perennial crop hindered by long reproducing cycle and plant size. MAS allows to accelerate the breeding process through the selection of cultivars with high-quality features and considerable mildew resistance characteristics (Bundessortenamt 2008). Recent research has led to the genetic identification of different grapevine DM resistance loci, so-called *Rpv* (resistance to *P. viticola*) (Table 1.2). An interesting case concerns *Rpv3* locus

that is responsible for the onset of a hypersensitive response (HR) at the infection sites within 2 days post inoculation (dpi) into the hybrid Bianca. Localised necrosis was the earliest phenotypic difference compared to susceptible individuals, it did not halt pathogen growth, but it was associated with a significant reduction of pathogen performance and disease symptoms from 3 to 6 dpi (Bellin et al. 2009). HR against *P. viticola* in grapevines carrying the *Rpv3* locus was associated to changes in expression of 33 genes, particularly the host reaction relied on transcriptional induction of the HR-associated gene HSR1 and salicylic acid-induced pathogenesis-related (PR) genes PR-1 and PR-2 during the initial 24–48 h post-inoculation (Casagrande et al. 2011). However, Peressotti et al. (2010) demonstrated that a *P. viticola* isolate (isolate SL) specifically overcomes *Rpv3* resistance, providing a putative example of emergence of a resistance-breaking isolate in the interaction between grapevine and *P. viticola*, and showing that a single resistance locus may not be sufficient for a durable resistance. Since the “pyramiding” of different resistance loci was described (Eibach et al. 2007), new cultivars were obtained with the breeding programs. The hybrid Solaris is an example of crossing between *V. vinifera* species and the Asian species *V. amurensis*, it presents the *Rpv10* locus (Schwander et al. 2012). This locus confers DM resistance accompanied by necrosis (Boso and Kassemeyer 2008), callose deposition (Gindro et al. 2003) and stilbene accumulation (Gindro et al. 2006, Pezet et al. 2004) as activated defence mechanisms. Schwander et al. (2012) analysed a population derived from a cross between grapevine breeding line Gf.Ga-52-42 and Solaris consisting of 265 F1-individuals, genetically mapped using SSR markers and screened for DM resistance. The F1 sub-population which contains the *Rpv10* as well as the *Rpv3* locus, showed a significantly higher degree of resistance, indicating additive effects by pyramiding of resistance loci.

Among genotypes derived from Solaris, the half-sibs Cabernet Cortis (Cabernet Sauvignon x Solaris) and Muscaris (Moscato bianco x Solaris) inherited the *Rpv10* - *Rpv3* loci and the *Rpv10* locus, respectively (*Vezzulli, pers. comm*.)

Table 1.1. Known and available grapevine genetic profiles.

Taxon	Number of genotypes	Molecular markers (Mm)[1]	Mm reference literature	Website	Link
Vitis vinifera	N.D.	9 SSRs	OIV 801, 802, 803, 804, 805, 806, 807, 808, 809	VIVC	http://www.vivc.de/
	68	8 SSRs	Bowers et al. 1999, This et al. 2004	GRIN	http://www.ars-grin.gov/
	2500	13 SSRs	Bowers et al. 1999, This et al. 2004, Crespan 2003, Welter et al. 2007	CREA-VIT unpub.	http://catalogoviti.politicheagricole.it/catalogo.php
Vitis hybrid	N.D.	9 SSRs	OIV 801, 802, 803, 804, 805, 806, 807, 808, 809	VIVC	http://www.vivc.de/
	788	8 SSRs	Bowers et al. 1999, This et al. 2004	GRIN	http://www.ars-grin.gov/
	200	13 SSRs	Bowers et al. 1999, This et al. 2004, Crespan 2003, Welter et al. 2007	CREA-VIT unpub.	
Vitis spp.	3355	9 SSRs	OIV 801, 802, 803, 804, 805, 806, 807, 808, 809	VIVC	http://www.vivc.de/
	N.D.	8 SSRs	Bowers et al. 1999, This et al. 2004	GRIN	http://www.ars-grin.gov/
	3500	13 SSRs	Bowers et al. 1999, This et al. 2004, Crespan 2003, Welter 2007	CREA-VIT unpub.	

[1] Mm detailed list:

9 SSRs: VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, VrZAG79, VVMD25, VVMD28, VVMD32

8 SSRs: VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, VrZAG79, VVMD31, VVMD34

Table 1.2. The QTLome of the grapevine downy mildew resistance trait (information derived from and integrated based on VIVC).

Symbol	Associated marker	Chromosome	Parental genotypes	Genotype of origin	Resistance source species	Reference
Rpv1	VVIb32	12	Syrah x 28-8-78	28-8-78	*M. rotundifolia*	Merdinoglu et al. (2003)
	VMC1g3.2 VMC8g09		Kismish Vatkana x VRH30-82-1-42	VRH30-82-1-42	*M. rotundifolia*	Katula-Debreceni et al. (2010)
Rpv2		18	Cabernet Sauvignon x 8624	8624	*M. rotundifolia*	Wiedermann- Merdinoglu et al. (2006)
Rpv3-1 (=*Rpv3* $^{299-279}$)	UDV-112	18	Regent x Lemberger	Regent		Welter et al. (2007)
	UDV-305 VMC7f2		Chardonnay x Bianca	Bianca		Bellin et al. (2009)
	VMC7f2		Regent x RedGlobe	Regent		van Heerden et al. (2014)
	UDV305 UDV737			Seibel 4614	*V. rupestris*	Di Gaspero et al. (2012)
	GF18-06 GF18-08		GF.GA-47-42 x Villard blanc	Villard blanc	*V. rupestris*	Zyprian et al. (2016)
Rpv3-2 (=*Rpv3* $^{null-297}$)	UDV305 UDV737			Munson (Jaeger 70)	*V. rupestris* or *V. lincecumii*	Di Gaspero et al. (2012)
	GF18-06 GF18-08		GF.GA-47-42 x Villard blanc	GF.GA-47-42	*V. rupestris* or *V. lincecumii*	Zyprian et al. (2016)
Rpv3-3 (=*Rpv3* $^{null-271}$)	UDV305			Noah	*V. labrusca* or *V. riparia*	Di Gaspero et al. (2012)
	UDV737		Merzling x Teroldego	SV5-276		Vezzulli et al. (in preparation)
Rpv3 $^{321-312}$	UDV305 UDV737			Noah	*V. labrusca* or *V. riparia*	Di Gaspero et al. (2012)
Rpv3 $^{361-299}$	UDV305 UDV737			Ganzin	*V. rupestris*	Di Gaspero et al. (2012)
Rpv3 $^{299-314}$	UDV305 UDV737			Ganzin	*V. rupestris*	
Rpv3 $^{null-287}$	UDV305 UDV737			Bayard (Couderc 28-112)	*V. rupestris* or *V. labrusca*	

Rpv4	VMC7h3 VMCNg2e1	4	Regent x Lemberger	Regent		Welter et al. (2007)
Rpv5	VVIo52b	9	Cabernet Sauvignon x *V. riparia* 'Gloire de Montpellier'	Gloire de Montpellier	*V. riparia*	Marguerit et al. (2009)
Rpv6	VMC8G9	12	Cabernet Sauvignon x *V. riparia* 'Gloire de Montpellier'	Gloire de Montpellier	*V. riparia*	Marguerit et al. (2009)
Rpv7	UDV-097	7	Chardonnay x Bianca	Bianca		Bellin et al. (2009)
Rpv8	Chr14V015	14	*V. amurensis* 'Ruprecht' x *V. amurensis* 'Ruprecht'	Ruprecht	*V. amurensis*	Blasi et al. (2011)
Rpv9	CCoAOMT IN0006 SSCP	7	Moscato Bianco x *V. riparia* 'WR63'	Wr63	*V. riparia*	Moreira et al. (2011)
Rpv10	GF09-46	9	Gf.GA-52-42 x Solaris	Solaris	*V. amurensis*	Schwander et al. (2012)
Rpv11	VVMD27 CS1E104J11 F VCHR05C	5	Regent x Lemberger	Regent		Fischer et al. (2004)
			Chardonnay x Bianca	Chardonnay		Bellin et al. (2009)
			Gf.GA-52-42 x Solaris	Solaris		Schwander et al. (2012)
Rpv12	UDV-014 UDV-304 rgvvin180 UDV-370	14	99-1-48 x Pinot noir	99-1-48	*V. amurensis*	Venuti et al. (2013)
			Cabernet Sauvignon x 20/3	20/3		
Rpv13	VMC1G3.2	12	Moscato Bianco x *V. riparia* 'WR63'	Wr63	*V.riparia*	Moreira et al. (2011)
Rpv14	GF05-13 VMC9b5 UDV111	5	Gf.V3125 x Börner	Börner	*V. cinerea* 'Arnold'	Ochssner et al. (2016)
Rpv15		18	*V. piasezkii* (DVIT2027) x F2-35	*V. piasezkii* (DVIT2027)	*V. piasezkii*	Pap et al. (in preparation)
Rpv16						Pap et al. (in preparation)

1.4.1.4.2. Downy mildew

In the grapevine-pathogen interaction, a pivotal role is also played by the DM pathovar. Effective control is mainly based on anticriptogamic treatments, although resistant strains to a number of fungicides have been reported. The resistance to chemical and organically-based fungicides has been used to characterize *P. viticola* isolates (Baudoin et al. 2008; Matasci et al. 2008). The study of this resistance transmission represents the earliest report about the inheritance of a phenotypic trait in *P. viticola* (Gisi et al. 2007). Subsequently, Blum et al. (2010) described for the first time the molecular mechanism of resistance to a carboxylic acid amide (CAA) fungicide. Sequencing of the *CesA* genes in a CAA-resistant and -sensitive field isolated strains identified five SNPs affecting the amino acid primary sequences of the cellulases; moreover one recessive mutation in *PvCesA3* causes inheritable resistance to the CAA fungicide mandipropamid.

As regarding the genetic characterization of *P. viticola* isolates, several genotyping studies rely on SSRs. Gobbin et al. (2003a) developed four co-dominant, neutral, highly reproducible and polymorphic microsatellite markers, which revealed different degrees of polymorphism within several oil spots (disease symptoms) collected from an infected vineyard. SSRs allow the high throughput analysis of DM epidemics and the investigation of within- and among-population genetic structure of *P. viticola* worldwide (Gobbin et al. 2003b, Gobbin et al. 2006, Koopman et al. 2007, Mochizuki et al. 2012, Yin et al. 2014). In contrast to theories which propose a massive vineyard colonization by one genotype and long-distance migration of sporangia, Rumbou and Gessler (2004) and Gobbin et al. (2005) found that epidemics of DM are caused by the interaction of several genotypes, each causing limited (or a few) lesions whilst a predominant genotype spreads stepwise at plot-scale. Additional seven SSR polymorphic loci were obtained from an enriched partial genomic library; cross-amplification tests on three closely related taxa indicated that two of these loci could be used in other oomycetes, proving to be useful for population genetic analysis across species (Delmotte et al. 2006). Later, 31 microsatellite markers, developed from microsatellite-enriched and direct shotgun pyrosequencing libraries of *P. viticola*, were optimized for population genetics applications (Rouxel et al. 2012).

Lately, Delmotte et al. (2011) developed a battery of 34 new SNP markers from an expressed sequence tag (EST) library of *P. viticola*, providing useful additional genetic tools for population genetic studies of this important agronomic species. Based on four internal transcribed spacer (ITS) regions, Rouxel et al. (2013 and 2014) combined a phylogenetic and morphological approach with cross-pathogenicity tests and large-scale sampling to

investigate host plant specialization and host range expansion in grapevine DM. By cross-inoculation experiments, the recent host range expansion of *P. viticola* from wild to cultivated grapevines was reconstructed, showing that it was accompanied by an increase in aggressiveness of the pathogen.

These overall results will have important implications for viticulture, including breeding for resistance and disease management. Most recently, the *P. viticola* draft genome sequence have been released (Dussert et al. 2016), allowing the development of new molecular markers and also the increase in knowledge about DM genes. DM gene prediction and annotation can take advantage of the sequenced genomes within the *Phytophtora* genus, an oomycete phylogenetically very close to *Plasmopara* (Tyler et al. 2006, Haas et al. 2009, Feau et al. 2016). This milestone opens the way towards the full understanding of the pathogen itself and in the grapevine-*P. viticola* interaction.

1.4.2. Transcriptomics and Proteomics

1.4.2.1. Grapevine

Gene expression analyses on grapevine and DM interaction have been largely carried out with a targeted approach by quantitative reverse transcription Polymerase Chain Reaction (RT-qPCR) on candidate genes. The gene selection is based on physiological, histochemical or biochemical observations and on sequence homology with defence-related genes of other plant species, such as genes encoding pathogenesis related (PR) proteins or enzymes implicated in the phenylpropanoid biosynthesis (Kortekamp 2006, He et al. 2013, Casagrande et al. 2011, Banani et al. 2014, Yu et al. 2016). This targeted approach highlighted stronger and faster up-regulation of defence-related genes (e.g. PR, callose synthase and enhanced disease susceptibility genes) in resistant as compared with susceptible genotypes in response to DM (Kortekamp 2006, He et al. 2013, Casagrande et al. 2011, Yu et al. 2016). Moreover, modulation of some key genes (e.g. flavonol synthase, leucoanthocyanidin dioxygenase and HR-associated gene) was observed exclusively in resistant genotypes after DM infection, suggesting the activation of specific resistance processes (Kortekamp 2006 and Casagrande et al. 2011). High throughput transcriptomic technologies allowed a better understanding of the transcriptional regulations of the grapevine response to DM, thus pinpointing key defence regulators to be used as candidate markers of grapevine resistance (Table 1.3A). A limited transcriptional modulation was detected in susceptible genotypes, reflecting the activation of an abortive defence response

(Polesani et al. 2010). Specifically, susceptible grapevines react to DM infection with a global down-regulation of photosynthesis-related processes and inadequate upregulation of genes encoding PR proteins, enzymes of phenylpropanoid pathways and regulators of response to stimuli (Polesani et al. 2008, Legay et al. 2011, Perazzolli et al. 2012, Vannozzi et al. 2012). On the other hand, resistant grapevines are characterized by the strong expression of stress- and defence-related genes before inoculation (Figueiredo et al. 2008) and by the rapid activation of defence and secondary metabolic processes after inoculation (Polesani et al. 2010, Wu et al. 2010, Malacarne et al. 2011, Figueiredo et al. 2012). Interestingly, the response of resistant genotypes involved the specific modulation of genes encoding components of signal transduction cascades, markers related to HR, and genes implicated in stilbene and defence hormone biosynthesis. All these genes are not modulated in susceptible genotypes and can be therefore associated to the early perception of the invading pathogen and to the activation resistance mechanisms (Polesani et al. 2010, Malacarne et al. 2011, Figueiredo et al. 2012). The transcriptional response to virulent and avirulent *P. viticola* strains confirmed the specific activation of defence-related transcription factors, *PR* genes, and secondary metabolic processes (Li et al. 2015). Interestingly, defence processes, specifically implicated to contrast DM in resistant cultivars, can be primed in susceptible genotypes by inoculation/treatments with resistance inducers, such as a beneficial microorganism (namely *Trichoderma atroviride* T39) and laminarins (Perazzolli et al. 2012, Gauthier et al. 2014). Transcriptional response of grapevine to DM was mainly studied on leaves but to date, no transcriptomic study was carried out on the inflorescences.

In addition to gene expression analyses, proteomic studies shed light into the plant-pathogen interaction (Table 1.3B). Proteomic studies demonstrated increases in abundance of grapevine defence-related proteins at 24 and 96 hpi, suggesting a transient breakdown in defence responses at 48 hpi associated to the onset of disease development (Milli et al., 2012). Furthermore, susceptible grapevine leaves accumulated allergenic defence-related proteins (PR-2 and β-1,3-glucanases; Rossin et al., 2015) and two glycoprotein implicated in the DM-induced deregulation of stomata during compatible interaction (Guillier et al., 2015). Interestingly, changes in the leaf proteome differ in resistant and susceptible *V. amurensis* varieties, demonstrating a specific increase in abundance of PR-10 and a decrease of photosynthesis proteins and ATP synthase, in a tentative to restrict DM infection (Xu et al., 2015). Grapevine genetic backgrounds containing the *Rpv1* and *Rpv3* pyramided resistance loci, are recognizable for the high accumulation of proteins associated to the redox, protein and energy metabolism, as well as stress and defence response

(Nascimento-Gavioli et al., 2016). A coordinated modulation of primary metabolisms specified processes of pyramided resistance and glycolytic pathways increased at late infection stages possibly to cope with the energetic demand of the plant defence (Nascimento-Gavioli et al., 2016). Likewise, induction of grapevine resistance by leaf inoculations with *T. atroviride* T39 primed changes in abundance of proteins related to response to stress and redox homeostasis, indicating stimulation of resistance processes (Palmieri et al., 2012). Protein modifications are key processes of the plant immune system to rapidly perceive pathogen infection and properly activate the defence reaction (Tena et al. 2011). Specifically, compatible interaction is associated to phosphorylation changes of proteins related to photosynthetic processes and protein metabolism, while DM resistance induced by *T. harzianum* T39 revealed phosphorylation changes in signal transduction cascades, hormone signalling, and gene expression regulations (Perazzolli et al., 2016).

Not only the host reaction plays a crucial role in the development of a compatible or incompatible interaction, but also the pathogen processes should be carefully taken into account. Pathogens rapidly evolve and can easily overcome plant resistance mechanisms (Jones and Dangl 2006); it has already been isolated a *P. viticola* strain able to overcome the *Rpv3* barrier in the hybrid Bianca (Peressotti et al. 2010). This highlights the necessity to clarify the genetic determinants of *P. viticola* virulence.

Table 1.3. Overview of transcriptomic and proteomic studies to identify candidate marker genes for DM resistance.

	Grapevine taxon	Grapevine genotype (clone)	Response[1]	Time point (hpi)[2]	Analysis[3]	Reference
A	*V. vinifera*	Riesling	S	2-3 weeks	cDNA-AFLP	Polesani et al, 2008
	V. vinifera	Chasselas	S	24	SSH	Legay et al., 2011
	V. vinifera	Pinot Noir	S	0, 24 and 48	RNA-Seq	Vannozzi et al., 2012
	V. amurensis	Zuoshan-1	R	24 h for 9 days	RNA-Seq	Wu et al., 2010
	V. vinifera *Vitis* hibrid	Trincadeira Regent	S/R	0	Microarray	Figueiredo et al., 2008
	V. vinifera *V. riparia*	Pinot Noir Gloire de Montpellier	S/R	12 and 24	Microarray	Polesani et al, 2010
	V. vinifera *Vitis* hibrid	*Pinot Noir* F1 21/66	S/R	0, 12, 24, 48 and 96	cDNA-AFLP and microarray	Malacarne et al., 2011
	V. vinifera *Vitis* hibrid	Trincadeira Regent	S/R	0, 6 and 12	Microarray	Figueiredo et al., 2012
	V. amurensis	Shuanghong	S/R	12, 24, 48 and 72	RNA-Seq	Li et al., 2015
	V. vinifera	Pinot Noir	S/IR	0 and 24	RNA-Seq	Perazzolli et al., 2012
	V. vinifera	Marselan	S/IR	12	Microarray	Gauthier et al., 2014
B	*V. vinifera*	Pinot Noir	S	24, 48 and 96	Proteomics	Milli et al., 2012
	V. vinifera	Marselan	S	144	Proteomics	Guillier et al., 2015
	V. vinifera	Pinot Noir	S	96	Proteomics	Rossin et al., 2015
	Vitis hibrid	-	R	24, 48 and 96	Proteomics	Nascimento-Gavioli et al., 2016
	V. amurensis *V. amurensis*	Shuangyou Shuanghong	S/R	0, 24, 72, 120 and 168	Proteomics	Xu et al., 2015
	V. vinifera	Pinot Noir	S/IR	0 and 24	Proteomics	Palmieri et al., 2012
	V. vinifera	Pinot Noir	S/IR	0 and 24	Phospho proteomics	Perazzolli et al., 2016

[1] Grapevine response to DM analysed by transcriptomics (A) and proteomics (B): response of susceptible genotypes (S), comparison of susceptible and resistant genotypes (S/R), and response of susceptible genotypes treated and not with resistance inducers (S/IR).
[2] Time point/s analysed after *P. viticola* inoculation expressed as hours post inoculation (hpi), except for Polesani et al. (2008), Wu et al. (2010).
[3] Details of the transcriptomic [amplified fragment length polymorphism (AFLP)-based transcript profiling (cDNA-AFLP), suppression subtractive hybridization (SSH), microarray or RNA-Seq] and proteomic analysis.

1.4.2.2. Downy mildew

The majority of the studies done until recently to unravel the molecular basis of the interaction between grapevine and *P. viticola* depicted this interaction mainly from the host point of view. Very few investigations describe the pathogen side as a consequence of its obligate lifestyle. And yet the identification of the pathogen effectors, and the elucidation of the way in which they can break down plant defences, represents an essential step in understanding the biology of the interaction and in planning the breeding programs for disease resistance. The first attempts done led to the identification of single or few *P. viticola* genes potentially involved in pathogenesis. The pionerist study by (Werner et al. 2002) describes two chitin synthases genes (*PvCHS1* and *PvCHS2*) differentially expressed during pathogen development. *PvCHS1* is constitutively expressed, also in the leaves showing symptoms of the disease, suggesting a link with the phase of intercellular growth of the hypha. On the contrary, *PvCHS2* is specifically expressed in sporangiophores and sporangia, and therefore probably mainly involved in the phase of dissemination of the infection. Blum et al. (2010) identified a family of four cellulose synthase genes potentially playing a role in host infection, since it was demonstrated that cellulose biosynthesis has an essential role in *P. infestants* pathogenesis (Grenville-Briggs et al., 2008). Luis et al. (2013), established the putative key steps of a successful colonization and cloned three genes theoretically involved in them, and potentially playing a role in pathogenesis: a NADH-ubiquinone oxidoreductase (PvNuo), which could be important for the capacity of the encysted zoospore to produce energy before the establishing of the infection; a laccase (PvLac), possibly contributing to the infection through its detoxifying activity; an invertase (PvInv), which could be involved in the uptake of the nutrients from the host.

The first large-scale investigation aimed to identify grapevine and *P. viticola* genes expressed during infection was performed by Polesani et al. (2008), which carried out a large-scale cDNA-AFLP analysis on infected grapevine leaves at the oil spot stage. Besides grapevine genes up- and down-regulated during infection, this work identified several pathogen transcripts, of which nine expressed in infected tissues and not in sporangia, suggesting a putative involvement in the infection process. In the last few years, with the introduction of next-generation sequencing technologies, the knowledge on oomycete effectors is greatly and rapidly improving. One of the first attempts to improve the public genomic resources available for *P. viticola* was carried out by Mestre et al. (2012), through the construction of cDNA libraries from *in vitro* germinated zoospores and infected grapevine leaves. Their analysis allowed the identification of genes potentially involved in the

pathogenic process, such as protein inhibitors, secreted hydrolytic enzymes, elicitor-like proteins and effectors. The expression profile of few of them (a INL11B-like elicitin, a protein with Kazal-like protease inhibitor fold and an RxLR protein) was characterized in different pathogen developmental stages.

The first complete and detailed analysis of the secretome of *P. viticola* during the infection process was performed by Yin et al. (2015). RNA-Seq analysis of cDNAs from infected leaves, at different time points, for three different pathogen isolates, led to the identification of over 500 potential secreted proteins, representing the full repertoire of apoplastic and cytoplasmic effectors. A first preliminary analysis performed on very few of them showed that the majority possesses the capacity to suppress programmed cell death. This result was confirmed in a following study (Xiang et al. 2016). A group of 23 putative RxLR effector candidates was chosen in the secretome for better characterization. Their expression pattern was studied, together with their subcellular localization and their capacity to repress cell death induced by various elicitors. It was demonstrated that quite all of them target the plant nucleus and act as suppressor of programmed cell death. Recently, a more general analysis of the effectors present in the genus *Plasmopara* has been performed (Mestre et al. 2016). The transcriptome of *P. halstedii* and *P. viticola*, from in vitro germinated zoospores and inoculated plant material, was used to create a *Plasmopara* species cDNA database. The full set of effectors used by both species, identified through the screening of the database, was compared within them and with seven sequenced oomycete species, representative of *Peronosporales* and *Albuginales*. This analysis allowed the identification of two classes of effectors: the species-specific ones, probably involved in host specificity, and the conserved ones, probably necessary for pathogen biology.

We are rapidly moving towards a comprehensive knowledge of the oomycete effectors. All the data produced until now highlight a really high complexity of the effector secretome, composed by several hundred proteins (Yin et al. 2015, Sharma et al. 2015). Future studies will require the identification of their host targets in order to understand the mechanisms of pathogenesis and disease and develop novel strategies to enhance grapevine resistance.

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CHAPTER 2

Development of a novel phenotyping method to assess downy mildew symptoms on grapevine inflorescences

Abstract

Grapevine downy mildew (DM), caused by the oomycete *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni, is one of the most important plagues affecting viticulture, especially in temperate rainy climates. *P. viticola* reduces fruit quality and yield, either by direct infection of berries or as a result of the reduction in photosynthesis and plant vigor caused by leaf infections. DM control is based on the repeated and massive use of fungicides, leading to problems such as environmental pollution, development of resistance and residual toxicity. The use of varieties showing durable resistance to DM is an alternative and promising strategy to control the disease. Nevertheless, most of the *in vitro* tests developed so far for DM resistance assessment are focused on leaf disc bioassays. This led us to consider that these tests might not always represent a proper evaluation and prediction of the disease symptoms extent on inflorescences/bunches and therefore on final yield and grape/wine quality. Therefore, based on the screening of nine *Vitis* hybrids, we developed a new *in vitro* phenotyping method to assess the disease extent on inflorescences at different phenological stages, along with a novel annotation descriptor (proposed OIV 453-1). Secondly, we combined this approach with the optimized leaf disc bioassay and found a general positive correlation between organ DM resistance phenotypes. Finally, we found that Cabernet Cortis could be a model to study divergent dual (on leaf and inflorescence) epidemics in downy mildew.

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2.1 INTRODUCTION

The evaluation of germplasm collections is a prerequisite for their employment in crop improvement. Vast genetic resources are available for crop plants, although to date few of them have been phenotypically well characterized. Precise and standardized phenotyping procedures of morphological and physiological - as well as abiotic/biotic stress tolerance and quality - traits have been always playing a crucial role in traditional breeding activities. Up to know, robust phenotypic data represent the major limiting resource to complement the current wealth of genomic information. The promise of using inexpensive sequencing technology to speed up plant breeding is being achieved with a vision of genomics-assisted breeding that will lead to hasty genetic gain for money-consuming and complex traits (Poland, 2015). Bust in plant phenomics, namely the study of plant growth, performance and composition, can sort out the phenotyping bottleneck. As regards herbaceous species, a wide range of tools is now accessible for high-throughput, fully automated and low-resolution phenotyping, facilitating the process of trait characterization, gene tagging and genotype development essential to release a new crop variety. By contrast, within woody species lower-throughput measurements with higher-resolution are feasible, affordable and thus desirable (Furbank and Tester, 2011).

Among fruit trees, grapevine is cultivated worldwide for the production of mostly wine, fresh fruit and raisins, and thus plays a pivotal role in the economy of many countries. Unfortunately, viticulture is endangered by numerous pathogens. Among those of primary importance, *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, is an obliged biotrophic pathogen which causes downy mildew (DM) to members of the *Vitaceae* family, in particular to the most cultivated species *Vitis vinifera* L. It was introduced in Europe in the 1870s, probably with the acquisition of American rootstocks resistant to *Phylloxera* used for grafting the susceptible European varieties (Viennot-Bourgin, 1949). Since then, grapevine DM has expanded across European regions and it is currently present in grape growing areas around the world, especially in temperate-humid climates.

All green plant tissues can be attacked. First symptoms generally come out as green-yellow lesions (also called oil spots) on the leaf surface. Suited conditions for sporulation are saturating humidity (>93%) and temperatures of 18-20°C. Sporulation can be observed on the abaxial side of the leaf and on the surface of tendrils, inflorescence and young berries. The oomycete overwinters as sexually produced oospore in fallen leaves and berries. In spring, with temperature above 10°C and rain precipitation, the oospores germinate and produce macrosporangia which release zoospores. Generally, 5–10 days after the infection,

depending on the temperature, DM produces sporangia containing asexually produced zoospores. Secondary disease cycles can take place under appropriate infection conditions which are similar to those suitable for primary infections (Gobbin et al., 2003). Depending on the environmental conditions, numerous clonal cycles may occur in one season leading to abrupt increase in disease severity with a disastrous impact on the yield. The organism is diploid in both sexual and asexual stages (Rumbou and Gessler, 2004).

Worldwide, the predominant strategy to control the disease is based on the use of pesticides. The repeated and massive application of chemical products not only entails huge expenses to grapevine production, but also leads to problems such as environmental pollution, development of resistance and residual toxicity. All these aspects foster human health concerns. Thus, the search for alternative approaches in the DM management is of paramount relevance for viticulture (Peressotti et al., 2010). The deployment of resistant *Vitis* hybrids showing durable resistance to DM is a promising strategy to control the pathogen (Topfer et al., 2011). Nevertheless, most of the *in vitro* tests developed so far for DM resistance assessment are focused on leaf disc bioassays (e.g. Staudt and Kassemeyer, 1995; Cadle-Davidson et al., 2008; Prajongjai et al., 2014) but not always represent a proper evaluation and prediction of the disease extent on grapevine inflorescence/bunch and therefore on final production and quality. Indeed the organ-specific nature of susceptibility to DM in some cultivars makes it complicated to deduce resistance in foliage to fruit and vice versa (Kennelly et al., 2005).

In this work we firstly developed a new *in vitro* phenotyping method (from infection to symptom evaluation) for DM resistance assessment on grapevine inflorescence, considering three different phenological stages. To obtain a practical and reliable assay to be employed mainly for breeding purposes, we compared this method with field performance (*in vivo*) of several genotypes. Thus, we identified the E-L 17 stage as the most reliable and suitable for *in vitro* evaluations. At this stage we screened all genotypes, in parallel with the established leaf disc bioassay (*in vitro*) (e.g. Peressotti et al., 2011), to compare the different pathogen responses between leaf and inflorescence collected from plants in an untreated field at Edmund Mach Foundation (FEM). We identified a case of DM divergent dual epidemics in Cabernet Cortis. Finally, we validated our results performing the same DM resistance assessment also on organs detached from fruiting cuttings grown in phytotron; our optimized fruiting cutting agronomic technique turned out to be crucial for the early evaluation of late-developing traits in grapevine, such as flowering.

2.2. MATERIALS AND METHODS

2.2.1. Plant material

The studied genotypes were chosen on the basis of their susceptibility, tolerance and resistance to DM as determined in previous field observations using the OIV 452 and OIV 453 descriptors for leaves and inflorescences, respectively (OIV, 2009). These two organs were collected from three plants of 9 *Vitis* hybrids and a *V. vinifera* variety grown in an untreated experimental field at FEM. In particular, inflorescences were harvested at three phenological stages (17, 25 and 29) of the Eichhorn-Lorenz (E-L) scale (Eichhorn and Lorenz, 1977); the phenological stage term is peculiar of grapevine and refers to the developmental stage (Mullins et al., 1992). In addition, for one relevant *Vitis* hybrid and one reference *V. vinifera* variety, the two organs were detached from fruiting cuttings grown under controlled conditions in phytotron (Table 2.1).

Table 2.1. The 11 studied grapevine genotypes, followed by their taxon, releasing country, FEM origin and *in vivo* preliminary information on downy mildew organ response level (mean of each studied phenological stage during 2012-2013).

Genotype (clone)	Taxon	Releasing country	FEM origin	E-L 17 stage		E-L 25 stage		E-L 29 stage		Preliminary DM response level in untreated field (*in vivo*)			
				L	I	L	I	L	I	Leaf (L)		Inflorescence (I)	
				OIV 452	OIV 453	OIV 452	OIV 453	OIV 452	OIV 453	Average OIV 452	Definition	Average OIV 453	Definition
Pinot Gris (SMA514)	*Vitis vinifera*	Italy	Field	3	4	3	1	2	1	2.67	Susceptible	2	Susceptible
MW14	*Vitis* hybrid	Austria	Field	5	3	3	3	3	1	3.67	Susceptible	2.33	Susceptible
16-02-102	*Vitis* hybrid	Italy/Austria	Field	3	5	3	1	1	1	2.33	Susceptible	2.33	Susceptible
Aromera	*Vitis* hybrid	Italy/Austria	Field	7	8	5	6	5	6	5.67	Tolerant	6.67	Tolerant
Bianca	*Vitis* hybrid	Hungary	Field	7	6	7	7	7	5	7	Tolerant	6	Tolerant
Bronner	*Vitis* hybrid	Germany	Field	9	9	9	9	9	8	9	Resistant	8.67	Tolerant
Jasmin8/1	*Vitis* hybrid	Hungary	Field	8	9	9	9	9	9	8.67	Resistant	9	Resistant
Muscaris	*Vitis* hybrid	Germany	Field	8	7	7	6	7	7	7.33	Tolerant	6.67	Tolerant
Regent	*Vitis* hybrid	Germany	Field	7	7	7	8	7	7	7	Tolerant	7.33	Tolerant
Cabernet Cortis	*Vitis* hybrid	Germany	Field/Phytotron	7	3	7	5	7	5	7	Tolerant	4.33	Susceptible
Pinot Noir (ENTAV115)	*Vitis vinifera*	France	Phytotron	-	-	-	-	-	-	-	-	-	-

2.2.2. Fruiting cutting production

In order to obtain fruiting cuttings, namely cuttings producing inflorescences and bunches within 4-6 months, a step-by-step protocol was optimized. Uniform hardwood cuttings were collected at pruning time from well-ripened dormant canes. The cuttings were sealed in plastic bags and stored under refrigeration (4°C) until required (Mullins, 1966). In the first step, called “pre-rooting”, the vine stock were cut at four or six nodes (N), based on the length of the internodes, and rehydrated in water for 24h at 30°C (Ollat et al., 1998); the bud from N0 was eliminated, treated with IBA (indol-3-butirric acid) 1000ppm for 30 seconds and potted in rock wool (Mullins and Rajasekaran, 1981). Cuttings were grown in a thermostatically controlled heated container (27°C at the bases of the cuttings) in a cold room (4°C). The moisture was kept by spraying water twice a week. Roots were produced by the cuttings, while buds remained dormant. After eight weeks the rooted cuttings were potted in the soil and transferred to the phytotron under controlled conditions: temperature at 27°C during day and 22°C during night, photoperiod 16/8h (light/dark) and RH 60%. At bud burst, leaves basal and adjacent to inflorescences were removed as soon as accessible and the shoot tip was excised (Antolin et al., 2010). These practices promoted inflorescence growth in a terminal position on the defoliated shoot. Thus, a lateral shoot was permitted to grow from one of the axillary buds proximal to the inflorescence. This shoot provided the 4-6 leaves to support the subsequent growth of the inflorescences. Per each genotype six fruiting cuttings were finally grown (Fig. S2.1).

2.2.3. Leaf disc *in vitro* bioassay upon downy mildew infection

Per each genotype, the fourth and fifth leaves under the shoot apex were detached from three individuals and rinsed with distilled water. Eight leaf discs of 2cm in diameter were excised from the six bulked leaves, paying attention to avoid veins, with a cork borer and plated onto wet paper in Petri dishes with the abaxial side up. Four leaf discs (replicates) were sprayed with *P. viticola* inoculum suspension at 1×10^5 sporangia ml^{-1} concentration (6,250 sporangia on each disc), while the remaining four were mock-inoculated (distilled water). *P. viticola* inoculum, named PVL-2012, derived from a collection on *V. vinifera* susceptible varieties in an untreated field. Petri dishes were incubated at 21°C in dark conditions for 48h in a growth chamber and then placed under light with a photoperiod of 16/8h (light/dark) for 7 days (Bellin et al., 2009). Susceptible Pinot leaf discs were employed as a positive control. Disease progress was monitored from 4 to 7 day post-infection (dpi)

and the degree of infection was quantified according to the level and the density of sporulation.

Inspiring to the OIV 452-1 descriptor (OIV, 2009), discs were scored with an overall parameter considering the disease symptom extent (Dis), and a specific parameter evaluating the density of sporangia (Sd) within leaf disc sectors yielding sporulation, irrespectively of the total area covered by sporulation (Bellin et al. 2009). Classes were named 1, 3, 5, 7 and 9 from the most susceptible to the totally resistant genotype. Necrosis presence (Nec) was also evaluated, based on an opposite scale (Fig. 2.1). Furthermore, annotation was optimized creating an integrated index (Int = √Dis*Sd) to resume more precisely the phenotype in a single value, thus facilitating comparisons and statistical analysis. Pictures of leaf discs were taken from 4 to 7 dpi using a digital camera (Canon EOS40D) in optimized artificial light conditions at constant focal length. Pictures were taken removing the lid from the Petri dish and each picture included the 4 leaf discs from the same genotype. Images were stored in a jpeg format and processed using the open source software ImageJ version 1.43q (http://rsb.info.nih.gov/ij/) with a semi-automatic quantification of sporulation (Peressotti et al., 2011). The results were plotted versus the number of dpi and finally, based on the latter, the AUDPC (Area Under Disease Progress Curve) was calculated, in order to assess also the progress of the disease during the time.

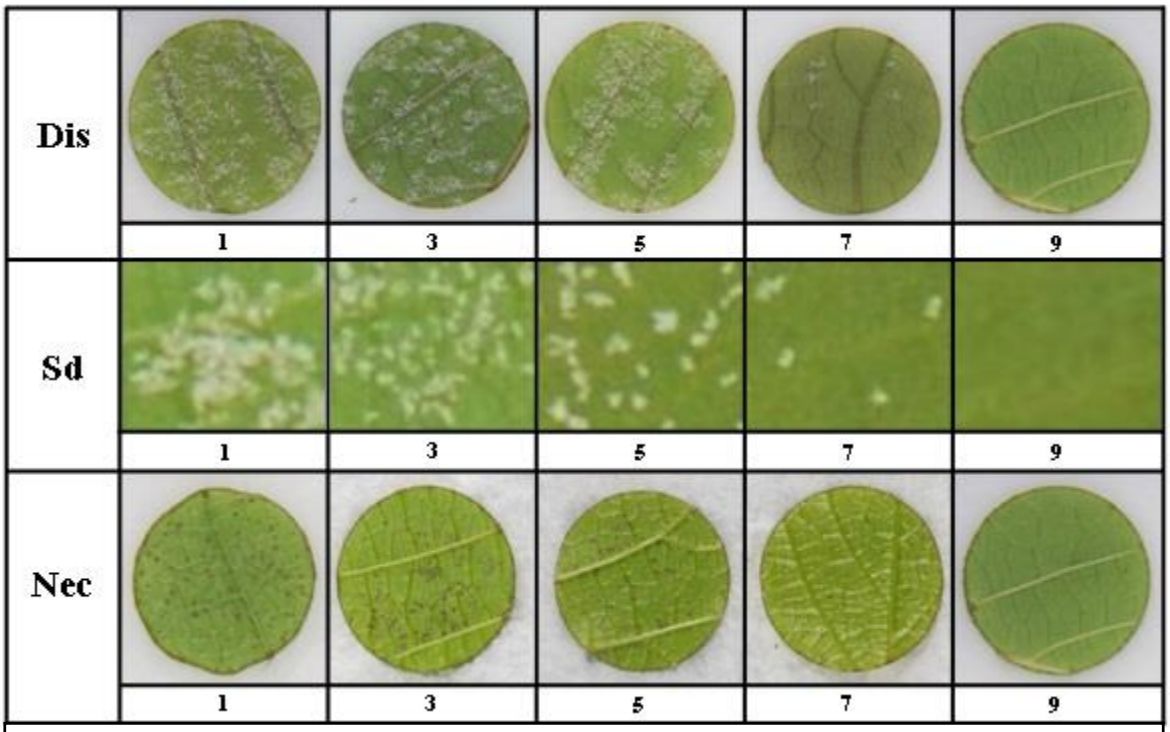


Fig. 2.1. Classes of Dis (disease extent), Sd (sporulation density) and Nec (necrosis presence) parameters. Dis and Sd classes ranged 1, 3, 5, 7 and 9 from the most abundant to the totally absent sporulation. Conversely, Nec classes ranged 1, 3, 5, 7 and 9 from the total absence to the most abundant presence.

2.2.4. Inflorescence *in vitro* bioassay upon downy mildew infection

To define the most effective infection method on inflorescence, two pilot studies were carried out during the spring 2014. Inflorescences, along with some leaves, of a susceptible genotype (Pinot Gris) were detached from plants at the E-L 17, 25 and 29 phenological stages grown in untreated field. They were divided in four groups with three replicates each: the first set was infected by spraying a *P. viticola* sporangia suspension, while the second one was mock-sprayed; the third group of inflorescences was infected by soaking in a *P. viticola* suspension, while the fourth one was mock-soaked. In particular, several combinations of soaking time and spore concentration were tested (data not shown). To confirm the presence of the pathogen in the tissues, a series of staining tests were carried out using a sporulated leaf disc as a reference.

In accordance to the most effective and successful infection trial, during the spring 2015 *P. viticola* inoculation assays were extended to an average of five (minimum three, maximum eight) inflorescences at each of the three studied phenological stages; they were

detached from each field- or phytotron-derived genotype of interest represented by three plants. The Pinot cultivar was considered as a positive control. An average of three (from two to four) inflorescences (replicates) were soaked with PVL-2012 suspension (1×10^4 sporangia ml^{-1}) for 2h to allow spore adhesion and germination, while the remaining were mock-inoculated. After infection, the inflorescences were placed in sterile boxes with the peduncle inserted in 1% agar covered with sterile wet filter paper. This step was essential to keep inflorescences alive. Then they were incubated at 21°C in dark conditions for 48h in a growth chamber and placed under light with a photoperiod of 16/8h (light/dark) for 7 days. Infected inflorescence tissues (flower clusters and rachis) were stained using the KOH-aniline blue fluorescence method (Díez-Navajas et al., 2007; Hood and Shew, 1996) with some modifications. To assess the KOH efficiency in discolouring plant pigments, flower clusters and rachis were individually and simultaneously incubated at 90°C in 1M KOH in distilled water, in 1.5 ml microtubes, for 5 min in a thermo-block (Eppendorf Thermomixer Comfort). The incubation was followed by three 15 min washes in distilled water and stained with 0.05 % aniline blue in 0.067M K_2HPO_4 (pH 9–9.5). The samples were examined under blue/violet light with a fluorescence microscope (Leica LMD 7000, excitation wavelength 400–440 nm, emission wavelength 475 nm).

Visual observations and pictures of DM symptoms were carried out at 2 and 6dpi using a stereomicroscope (Leica MZ16F). Two parameters derived from the OIV 453 descriptor, for grape cluster DM resistance in field (OIV, 2009), were assigned: Dis, distribution of *P. viticola* on the total length of the inflorescence, and Sd, sporulation density of *P. viticola* considering each single spot. Classes were named 1, 3, 5, 7 and 9 according to the degree of resistance, where the higher is the class the stronger is the resistance. As for leaf disc assessment, the Int (√Dis*Sd) index was introduced.

A new quantification method for a low number of sporangia ml^{-1} ($\leq 1.5 \times 10^4$) was developed, testing first leaf discs and then analysing inflorescences. Upon addition of 1 mL of dH_2O, each sample was mixed and inflorescence tissues were eliminated through filtration. Thus a first number of sporangia was estimated with Malassez's counting chamber. After centrifugation at 3,000 rpm for 10 min, 700 µL of dH2O were discarded and a second count of sporangia was performed.

2.2.5. Statistical analysis

Statistical analyses were carried out using in-house developed routines written in R language (R Development Core Team. R: A Language and Environment for Statistical

Computing. Vienna, Austria, 2009. http://www.R-project.org.). Comparisons of variable mean values between two groups of samples (or between a group and a reference value) were performed via the parametric t-test ($p<0.05$), after testing normality and homogeneity of variances (i.e. homoscedasticity) by Shapiro-Wilk test and Levene's test, respectively. In case of heteroscedasticity of the data, a modified t-test (Welch's t-test) which obviates to this problem was used, while in case of non-normality the non-parametric Wilcoxon Rank Sums test was employed. Uncertainties of mean values were computed as standard errors. Correlations were assessed via Pearson's correlation coefficients, also computing the relative p-values to determine significance ($p < 0.05$).

2.2. RESULTS

2.3.1. Field-derived leaf disc downy mildew resistance assessment

According to the preliminary definition of DM resistance level in untreated field (Table 2.1), upon leaf disc infection the susceptible genotypes had few points of infection (medium-high Dis) with low density of sporulation (medium-high Sd), while the tolerant/resistant ones were characterized by absence or almost of the disease (high Dis and Sd) at 4dpi. The situation resulted opposite for the necrosis presence: tolerant/resistant genotypes showed high levels (high Nec), while susceptible ones presented low levels (low Nec). Compared to 4dpi, at 7dpi the trend for susceptible genotypes was similar presenting strong disease sporulation and sporulation density (medium-low Dis and Sd). Indeed, the resistant genotypes had different values characterized by a weak disease sporulation and sporulation density, while the necrosis were subjected to a general increase. Mock samples had the same behaviour at 4dpi and 7dpi presenting score 9 for both Dis and Sd and 8 or more as Nec average value (Fig. 2.2). Moreover, linear correlations between the Dis parameter at 7dpi on leaf disc and the corresponding OIV 452 descriptor on foliage revealed that leaf disc bioassay results are significantly associated to the *in vivo* DM response at foliar level (Table 2.2A).

Based on the developed leaf disc Int index at 7dpi, all genotypes of interest were divided in 4 classes, following a parametric test ($p \leq 0.05$). An Int value higher than 7 defined the resistant genotypes (R), higher than 5 the mid-resistant ones (MR), lower or equal to 5 the mid-susceptible ones (MS), and lower than 2.5 the susceptible (S) ones (Table 2.3). No genotype fell into the S class; the positive control Pinot Gris resulted in MS class. At 4dpi (early stage of the pathogen development) all genotypes did not show any DM response

symptom. The leaf disc Dis and Sd parameter confirmed the Int-based classification (Table S2.1).

Development of the necrosis (Nec) at both 4dpi and 7dpi was not statistically significant on resistant genotypes compared to mock samples. Indeed, based on the calculated AUDPC, which resumes the progress of DM from 4dpi to 7dpi, a low but significant ($p \leq 0.05$) variation was detected among genotypes. In the MS genotypes the increase of sporulated surface was relevant; for example in the *V. vinifera* cv Pinot Gris it significantly increased from the 0.06% to the 11.08% ($p \leq 0.001$). In the MR genotypes there was a low but significant increase; for example in the *Vitis* hybrid Cabernet Cortis the sporulation trend was almost flat, from 0.01% to 1.05% ($p \leq 0.05$) (Fig. 2.3 and Fig. S2.2).

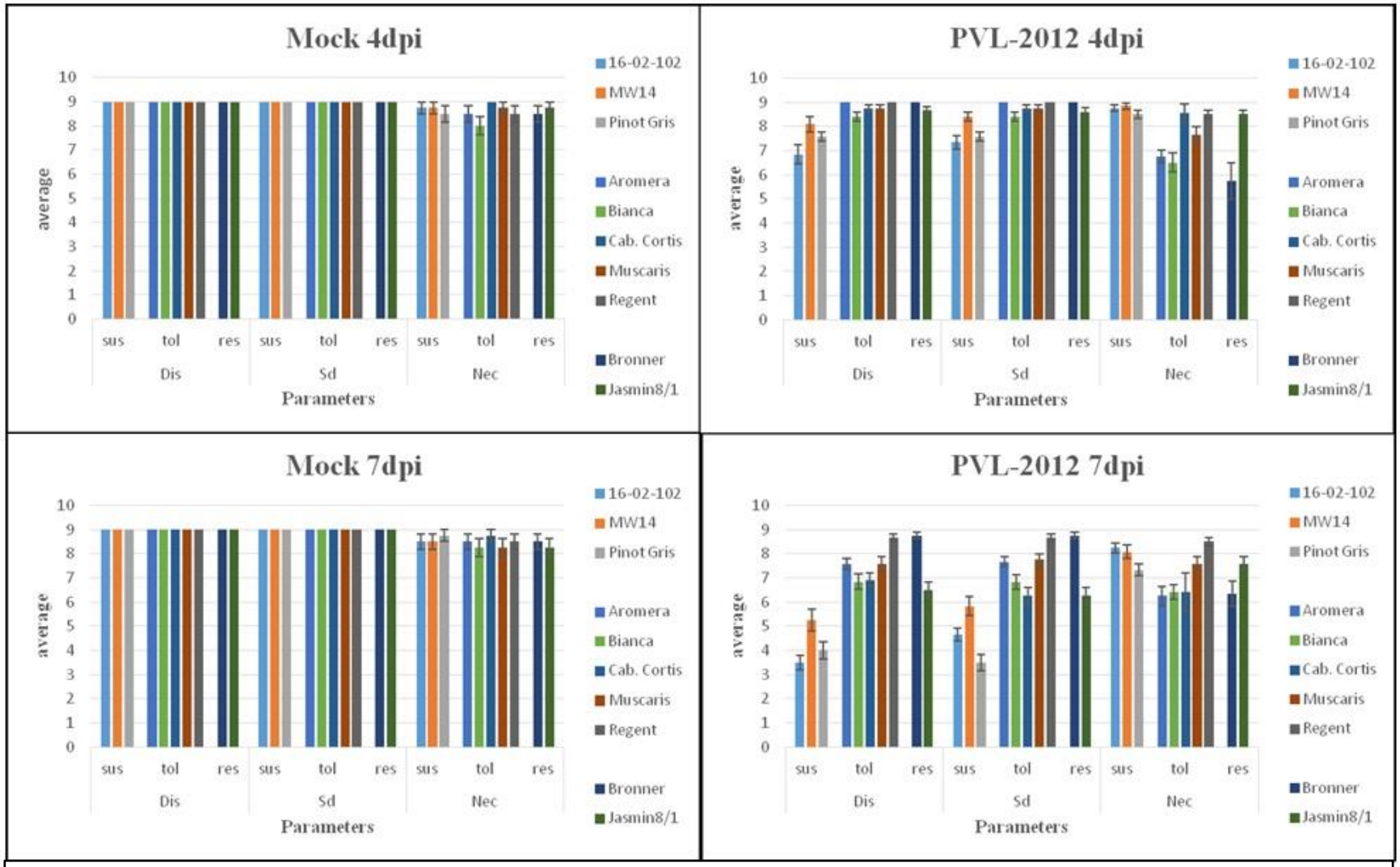


Fig. 2.2. Leaf disc *in vitro* bioassay results based on the average of the three evaluated parameters. dpi: days post-infection, Dis: disease extent, Sd: sporulation density, and Nec: necrosis.

Table 2.2. Correlations between the evaluated parameters on leaf (disc) and inflorescence at three phenological stages in all studied genotypes. Panel A: between the OIV 452/453 descriptors observed *in vivo* and the corresponding Dis parameters recorded *in vitro* at leaf and inflorescence level. Panel B: between the OIV 452 and the OIV 453 descriptors observed *in vivo* respectively in leaf and inflorescence (— = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, / = not calculated).

A

Infection condition	**Phenological stage**	**Organ**	*In vitro* (field-derived organs)					
			E-L 17		E-L 25		E-L 29	
			Leaf (disc)	Inflorescence	Leaf (disc)	Inflorescence	Leaf (disc)	Inflorescence
In vivo	E-L 17	Leaf	r = 0.85 (**)	/	/	/	/	/
		Inflorescence	r = -0.06 (—)	r = 0.92 (***)	/	/	/	/
	E-L 25	Leaf	/	/	r = 0.85 (**)	/	/	/
		Inflorescence	/	/	/	r = 0.29 (—)	/	/
	E-L 29	Leaf	/	/	/	/	r = 0.68 (*)	/
		Inflorescence	/	/	/	/	/	r = 0.29 (—)

B

Infection condition	**Phenological stage**	**Organ**	*In vivo*		
			E-L 17	E-L 25	E-L 29
			Inflorescence	Inflorescence	Inflorescence
In vivo	E-L 17	Leaf	r = 0.68 (*)	/	/
	E-L 25	Leaf	/	r = 0.93 (***)	/
	E-L 29	Leaf	/	/	r = 0.94 (***)

Table 2.3. Classification of the 11 studied genotypes into four DM response classes at leaf (disc) level, based on the integrated index scores. S: susceptible, MS: mid-susceptible, MR: mid-resistant, and R: resistant (— = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Genotype	**Threshold > 7**	**Threshold > 5**	**Threshold < or = 5**	**Threshold < 2.5**	**DM response level**
16-02-102	—	—	***	—	MS
Aromera	**	***	—	—	R
Bianca	—	***	—	—	MR
Bronner	***	***	—	—	R
Cabernet Cortis	—	***	—	—	MR
Jasmin8/1	—	***	—	—	MR
Muscaris	*	***	—	—	R
MW14	—	—	*	—	MS
Pinot Gris	—	—	***	—	MS
Regent	***	***	—	—	R

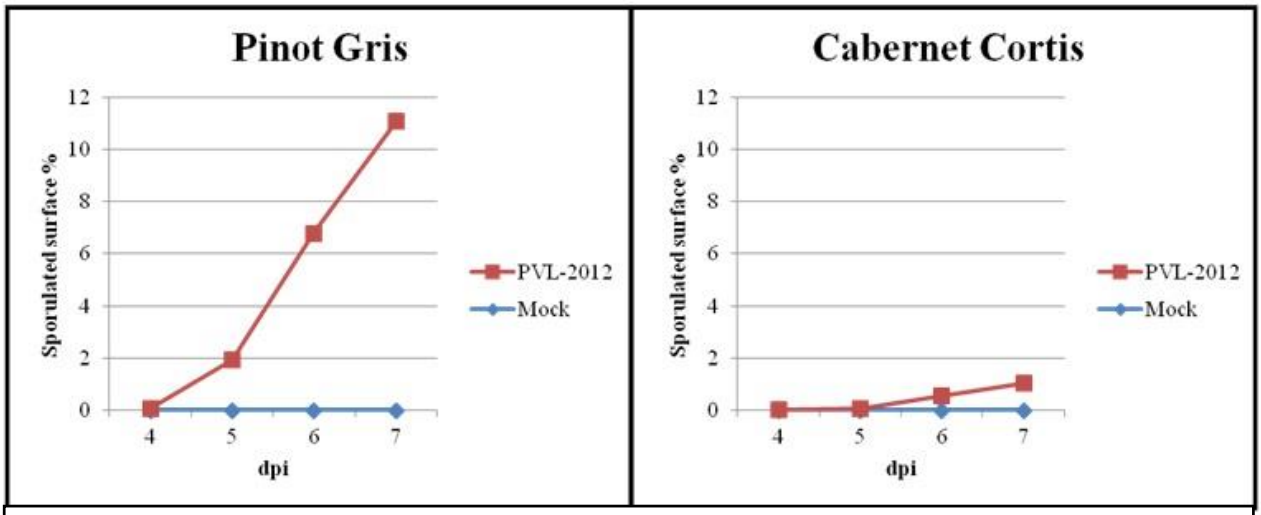


Fig. 2.3. Percentage of leaf disc sporulated surface on one mid-susceptible *V. vinifera* variety (Pinot Gris) and one mid-resistant *Vitis* hybrid (Cabernet Cortis) upon downy mildew (PVL-2012) infection versus mock-inoculation, at different days post-infection (dpi).

2.3.2. Field-derived inflorescence downy mildew resistance assessment

Upon the two pilot studies, the samples infected spraying the pathogen sporangia rotted very quickly, while the soaking method did not affect plant tissues. Therefore, the latter infection method was preferred for the following *P. viticola* inoculation assays. The phenotypic observations of the sporulation on inflorescences showed four satisfying combinations of soaking time and spore concentration. The one representing the right compromise between technical time employed and good level of sporulation on positive control was chosen as the best infection method (Table S2.2). The presence of the pathogen in the tissues of the positive control was confirmed by staining tests. At 6dpi in Pinot Gris the mycelium growth was widespread as well as the sporangia and spores were visible, while in the mock samples there was absence of sporulation (Fig. 2.4). Subsequently, this successful infection method was extended to field-derived inflorescences of all genotypes of interest at the three studied E-L phenological stages. Different degrees of disease symptoms were observed both on the total length of the inflorescence and on single spots. This led to the creation of a scale that is an adaptation of the *in vivo* OIV 453 descriptor in order to describe the different rates of downy mildew response upon an *in vitro* infection (Fig. 2.5).

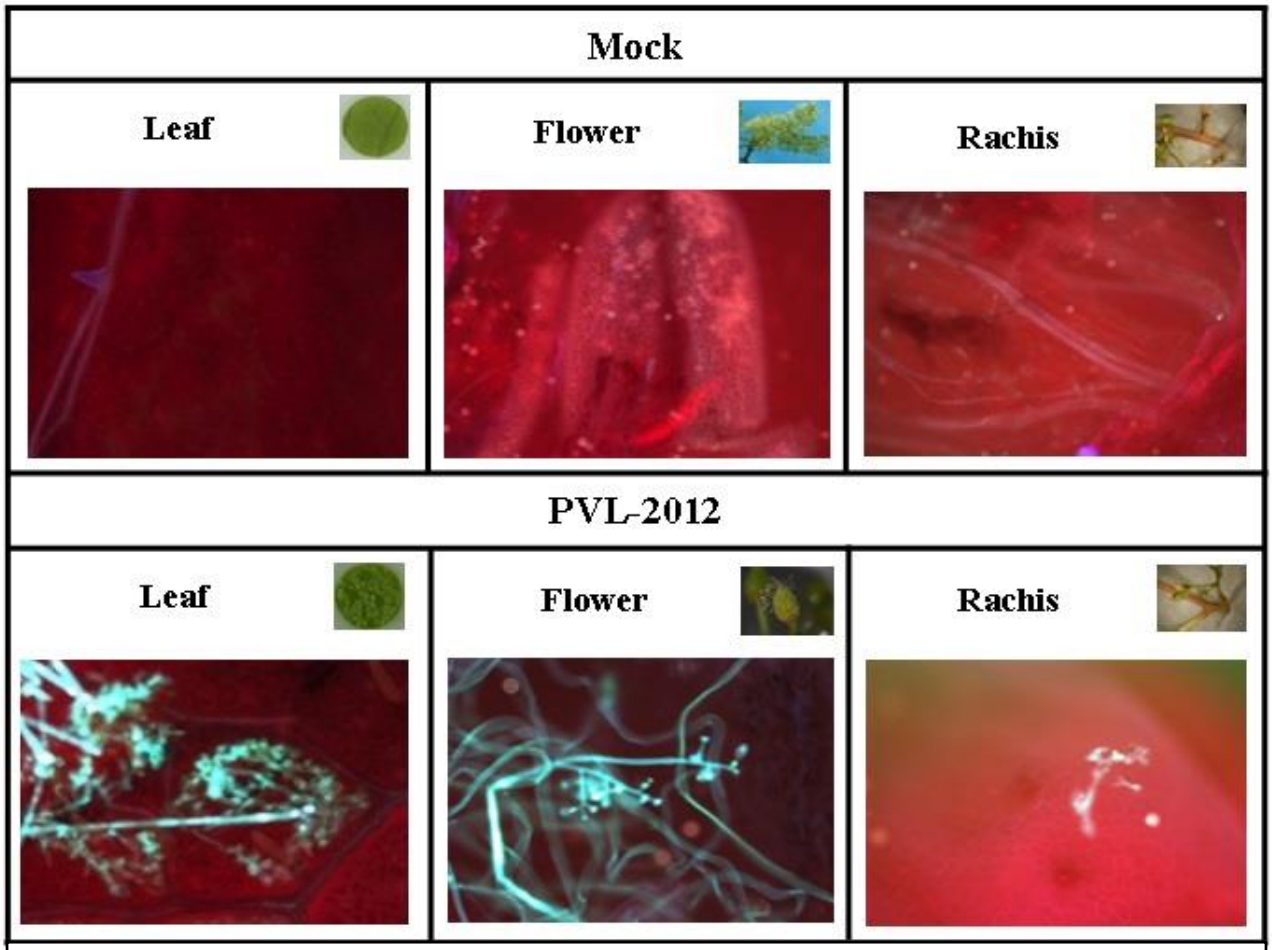


Fig. 2.4. Pilot study staining results on leaf disc, flower and rachis of Pinot Gris upon downy mildew (PVL-2012) infection and mock-inoculation at 6 dpi (top picture 5X zoom; bottom picture 20X zoom).

Based on the developed inflorescence Int index at 6dpi, it was not possible to perform a classification using a parametric test due to a lack of variance in most studied genotypes at each studied phenological stage. At the same time, it was relevant to define at which developmental stage the inflorescences were more reactive to the pathogen attack under *in vitro* conditions. Linear correlations between the inflorescence Dis parameter at 6dpi and the corresponding OIV 453 descriptor (preliminary data) demonstrated that inflorescence *in vitro* assay results significantly reflect the *in vivo* DM response at inflorescence level only at the E-L 17 phenological stage (Table 2.2A). Therefore, this developmental stage resulted to be the most robust and suitable for *in vitro* evaluations out of the three considered ones. Based on the latter DM response assessment, genotypes were assigned to classes following the four thresholds set for leaf discs. In particular, Aromera, Bronner, Jasmin8/1 and Muscaris revealed to be R, Bianca, Regent, 16-02-102, MW14 and Pinot Gris were MR,

while Cabernet Cortis resulted MS. As in the case of leaf disc bioassay, no genotype was classified as S. The inflorescence Dis parameter confirmed the Int-based classification, while Sd provided MW14 and Cabernet Cortis with inconsistency, ascribing them to the MR class instead of MS (Table S2.1).

Upon staining tests, in the inflorescence of MS genotypes, such as Cabernet Cortis, the hyphae and mycelium were recognizable and the sporangia and spores were clearly visible, while in MR genotypes, such as Regent, the mycelium and the sporangia were present but only in some spots. In the rachis of MS genotypes the germ tube under stoma and the sporangia were present, while in MR genotypes no structure attributable to the pathogen was present (Fig. S2.3). The number of sporangia counted at 6 dpi varies with the growth of the inflorescence based on the genotype. Between the first and the second count there was a significant linear correlation, respectively with $r = 0.71$, $r = 0.81$ and $r = 0.97$ ($p \leq 0.05$) (Fig. S2.4).

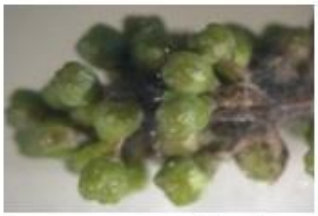
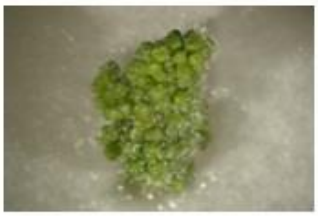
Dis	Sd
9  Absent sporulation	9  No sporulation density
7  Rare sporulation	7  Single spot sporulation
5  Widespread sporulation	5  Low dense sporulation
3  Abundant sporulation	3  Locally dense sporulation
1  Highly abundant sporulation	1  Very dense sporulation

Fig. 2.5. Classes of Dis (disease extent) and Sd (sporulation density) parameters. Class 9: absent sporulation on the overall inflorescence (no infected flower, n=0%) and no attack. Class 7: rare sporulation on the overall inflorescence (limited number of infected flowers, n<30%) and single spot attacks. Class 5: widespread sporulation on the overall inflorescence (fair number of infected flowers, 30%<n<50%) and low dense attacks. Class 3: abundant sporulation on the overall inflorescence (high number of infected flowers, 50%<n<80%) and locally dense attacks. Class 1: highly abundant sporulation (very high number of infected flowers, n>80%) and very dense attacks.

2.3.3. Comparison between field-derived leaf disc and inflorescence disease response

Linear correlations between leaf (disc) and inflorescence Int indexes (*in vitro*) confirmed that the E-L 17 stage (flower button formation) is the most robust and suitable for DM response assessment under *in vitro* conditions, being the only one out of three to present a significant positive correlation ($r = 0.62$, $p \leq 0.05$) between the two organs. This is in contrast to the natural (*in vivo*) infection condition where the two organ results showed a significant positive correlations at all three studied developmental stages (Table 2.2B). Focussing on this crucial phenological stage, we compared the leaf Int results with both inflorescence Dis and Sd in order to understand if they had a similar trend. A significant positive correlation was found in both cases ($r = 0.62$, $p \leq 0.05$). Indeed, the AUDPC leaf values did not significantly correlated with the inflorescence spore counts. According to the calculated Int standard errors, genotypes classified as MR or R at leaf level had also R inflorescences; genotypes with MS leaves presented S inflorescences except for Cabernet Cortis, carrying MR leaves and MS inflorescences, and unexpectedly Pinot Gris, displaying MS leaves and MR inflorescences (Table S2.3). Finally, linear correlation between leaf (disc) Dis parameter (*in vitro*) and inflorescence OIV 453 descriptor (*in vivo*) at the E-L 17 stage was not significant (Table 2.2A).

2.3.4. Phytotron-derived leaf disc and inflorescence downy mildew assessment

To verify the unexpected Pinot Gris DM response at inflorescence level and confirm that the hybrid Cabernet Cortis had a significantly different response between leaf (MR to DM) and inflorescence (MS to DM) also under controlled conditions, we detached organs from produced fruiting cuttings (E-L 17 stage) grown in phytotron. Thus, we screened them upon *P. viticola* infection and mock inoculation, according to the *in vitro* phenotyping methods employed for field-derived organs. As a positive control we used Pinot Noir which is well known to have both leaf and inflorescence susceptible to the pathogen attack.

Based on Int results at 7 dpi, Cabernet Cortis as well as Pinot Noir presented a significant ($p \leq 0.05$) induction upon DM infection at leaf level (infected vs mock), although they actually showed an Int value of 5.16 (MR) and 3.94 (MS), respectively. Nec and AUDPC values showed the same significant results. Analogously, at inflorescence level (infected vs mock) these genotypes presented a similar significant ($p \leq 0.05$) response to *P. viticola*,

although resulting to have MS (Int = 4.12) and S (Int = 2.24) inflorescences, respectively. Moreover, considering the comparison between infected organs within the same genotype, Cabernet Cortis presented a significant difference in DM response, while Pinot Noir did not show a significant response variation between foliage and inflorescences (Table 2.4A and Table S2.1).

At foliar level Cabernet Cortis and Pinot Noir showed a significantly different response upon DM infection, resulting to be MR and MS respectively according to their mean Int, in agreement also with AUDPC and Nec values. Concerning inflorescences, these two studied genotypes did not show significant differences, given their similar Int values ascribing them to the MS and S class, respectively (Table 2.4B and Table S2.1).

Finally, according to Int scores, field-derived leaf (disc) and inflorescence of Cabernet Cortis did not show significant differences in DM response from phytotron-derived organs. Indeed, Pinot did not show significant discrepancy at leaf level, while revealed a significance difference at inflorescence level between field-derived and phytotron-derived samples (Int = 6.20 and 2.23, respectively) (Table S2.1 and Table S2.4A and S2.4B).

Table 2.4. Comparisons between the evaluated parameters on leaf (disc) and inflorescence of genotype of interest. Panel A: different organs within the same genotype, Cabernet Cortis and Pinot Noir. Panel B: the same organ between Cabernet Cortis and Pinot Noir. Int: integrated index, Nec: necrosis, AUDPC: area under disease progress curve (– = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, / = not calculated).

A					
	Organ				
	Leaf (disc) (Infected vs Mock)			Inflorescence (Infected vs Mock)	Leaf (disc)-Inflorescence (Infected vs Infected)
	Parameter			**Parameter**	**Parameter**
Genotype	Int	Nec	AUDPC	Int	Int
Cabernet Cortis	***	***	***	**	*
Pinot Noir	***	***	***	***	–

B						
Genotype			Pinot Noir			
	Organ		Leaf (disc) (Infected)			Inflorescence (Infected)
		Parameter	Int	Nec	AUDPC	Int
Cabernet Cortis	Leaf (disc) (Infected)	Int	***	/	/	/
		Nec	/	**	/	/
		AUDPC	/	/	***	/
	Inflorescence (Infected)	Int	/	/	/	–

2.4. DISCUSSION

2.4.1. The *in vitro* foliage disease response variability

In agreement with the preliminary field observations, the present inoculation experiments carried out on leaf discs confirmed a different degree of susceptibility and resistance to *P. viticola* among genotypes. This observation is in agreement with a number of previous studies focused on the *in vitro* screening of inter-specific hybrids, highlighting that non-*vinifera* materials are not all tolerant or resistant to mildews (e.g. Staudt and Kassemeyer, 1995; Prajongjai et al., 2014). The leaf disc *in vitro* test was based on the fourth and five fully expanded leaf, which do not show ontogenic resistance, also termed age-related resistance (AAR), yet (Steimetz et al., 2012). In fact, the older leaves of the bottom of the grapevine shoots present a higher resistance to DM (Reuveni, 1998) and PM (Doster and Schnathorst, 1985) than the younger ones.

As concerning the employed phenotypic parameters, Int has been chosen as a leaf resuming factor of both disease symptom extent and density, providing a reliable genotype classification. Given the uniformity of the leaf disc surface, the Int, Dis (comparable to OIV 452-1) and Sd release a consistent class at 7dpi (data not shown). The Nec parameter was not considered a robust index of resistance because of its instability; in fact necrosis observation was more difficult at 7dpi, probably due to the growth of the sporangiophores and sporangia that partially cover the leaf disc surface; besides hypersensitive reaction, it can generally be associated to both to host and non host resistance and could be affected by various physiological factors (Bashir et al., 2013; Heath, 2000). This study was also characterized by the calculation of the AUDPC parameter, which resumes the disease progress from 4dpi to 7dpi, based on an image analysis optimized procedure. This semi-automatic screening method enabled to obtain a uniformly detected %spor (although underestimated for intrinsic reasons), representing an attempt to allow for standardized comparisons among different studies. The %spor, a measure of disease severity according to EPPO (OEPP/EPPO, 2001), represents a linker to compare current results with previous findings on leaf disc bioassay. At present, this method is established in terms of infection protocol, but is not uniform in term of annotation procedure. Imaging methodologies are currently under increasing use within the grapevine community committed in disease resistance assessment (e.g. Kicherer et al., 2015; Petrovic et al., 2014; Poutaraud et al., 2007), depending on the expansion of image-based phenotyping of plant disease symptoms (Mutka and Bart, 2015). For instance, image analysis-based techniques, such as imaging of chlorophyll fluorescence, have been applied to monitor the advancement of disease

symptoms at leaf level for some years (Scholes and Rolfe, 2009). Besides the phenomic screening for biotic stress tolerance, a range of imaging techniques are also being employed as rapid, non-destructive and reliable tool to record data for quantitative studies of complex traits related to the yield (Millan et al., 2016), growth and adaptation to abiotic stress (Li et al., 2014). Our study finally demonstrated that the *in vitro* leaf disc results (Dis) have a significantly positive correlation with the *in vivo* foliage disease response (OIV 452). This aspect confirms that the leaf disc bioassay is a robust predictor of field resistance/susceptibility at leaf level, as previously reported in literature (Boso et al., 2014; Brown et al., 1999; Sotolář, 2007). By contrast, our observed *in vitro-in vivo* correspondence is not consistent with the lack of correlation between leaf disc infection and natural infection in the vineyard reported by Cadle-Davidson (2008). This fact can be explained by their *in vitro* use of single-isolate inoculations compared to the actual inoculum derived from a mix of isolates collected in untreated vineyards. Actually, DM variability has been investigated from both the genetic (e.g. Schröder et al., 2011), the phenotypic (e.g. Gómez-Zeledón et al., 2013), and the combined (Delmotte et al., 2014) points of view.

2.4.2. The new *in vitro* inflorescence phenotyping method

Analogously to leaves, inflorescences showed a phenotypic variability range among genotypes upon DM *in vitro* infection. This observation is consistent with studies mainly reporting on field trials (e.g. Pacifico et al., 2013, Boso et al., 2004, Boso et al., 2011); in fact, the DM response on grapevine inflorescences has not been deployed *in vitro* or barely mentioned, without highlighting the protocol employed under controlled conditions. For instance, Gindro et al. (2012) observed successful infections on detached clusters of several cultivars at BBCH 53 (namely E-L 12) stage when functional stomata were present, while no infections were observed after this stage when stomata were closed-like. This is in agreement with our findings which revealed how the grapevine phenological stage is of paramount importance for the DM resistance *in vitro* assessment at inflorescence/bunch level. The significantly positive correlation between *in vivo-in vitro* results revealed that the most reliable and suitable stage is the E-L 17, corresponding to flower button formation. This stage is supposed to largely anticipate the onset of the ontogenic resistance against *P. viticola*, although this aspect is still uncertain. In fact, it has been suggested that this type of resistance is associated with loss of the infection court as stomata are converted to lenticels, but beginning time and seasonal variation in ontogenic resistance has remained unclear at flower/berry level. Particularly *in vivo*, the time of onset and following expression of this age-

related resistance to *P. viticola* may be affected by weather conditions and should be pondered in transferring results from a climatic area to another one (Kennely et al., 2005). Moreover, cultivar variation dramatically impacts on the ontogenic resistance degree. *P. viticola* infects only through colonization of stomata, which are known by several studies to vary in number on the berry surface at anthesis among cultivars. This micro-morphological difference could be responsible for the different pathogen penetration capability (e.g. Bessis, 1972, Bernard, 1977, Nakagawa et al., 1980).

Therefore, these considerations led us not to exclude the possibility that different genotype reactions at inflorescence level can be due to this issue, underlining the necessity and the relevance of *in vitro* test that partially gets rid of environmental effects and enhances the cultivar contribution, from both the genetic and the morphological point of view. Unlike evaluation in vineyard, at *in vitro* level no standardized protocol is available for inflorescence/bunch downy mildew resistance assessment. In the same vein of the corresponding OIV 452-1 on leaf discs (herein comparable to Dis), we first developed the putative OIV 453-1 descriptor (herein referred as Dis) on grapevine inflorescence that is currently under evaluation by the OIV agency (Vezzulli S., pers. comm.). Although the OIV code has not been worldwide adopted yet – it has a European preference – it has been recently translated in Chinese (http://www.oiv.int).

This novel protocol and proposed OIV descriptor represent a relevant breakthrough within the grapevine and also for the plant disease resistance phenotyping community. We also updated protocols for pathogen spore counting in case of small quantities. ImageJ or particle counters are reported to be efficient to differentiate susceptible from resistant genotypes or to investigate in detail high and medium susceptible ones (Peressotti et al., 2011; Delmotte et al., 2014). Unfortunately, in case of high resistant genotypes or small sporulated samples, as inflorescences, these tools are not the most reliable. In fact, the background noise is too high to allow a fine and precise resolution on few sporangia to count. To overcome this issue, we inspired from the protocol used by Weßling and Panstruga (2012) and developed a reliable and cost effective method to enable spore counts in case of low sporulation or small sampling material.

Unlike in leaf, Dis provided a different class information from Sd parameter, the latter being more impacting for the evaluation of DM response on inflorescence. This outcome could be ascribed to the lack of uniformity of the inflorescence surface compared to leaf disc. Int is in agreement with Dis which turned out to be the most reliable parameter for the DM resistance assessment at inflorescence level. Based on the latter, the inflorescence *in vitro*

assay results significantly reflect the *in vivo* DM response (OIV 453) at inflorescence level (E-L 17 stage) supporting the reliability of our novel phenotyping method. In particular, unlike in leaf, it is relevant to notice that *in vitro* tested field-derived inflorescences of Pinot Gris resulted less susceptible than the *in vivo* ones. This can be ascribed to the fact that grapevine organs, in particular inflorescence under growth, are challenged by abiotic and also biotic stress in untreated field and can be pre-alerted and thus respond with minor efficacy to the following *in vitro* assay (Bellin et al., 2009). In fact, this assumption is demonstrated by comparing field- and phytotron-derived *in vitro* results where Pinot Noir resulted fully susceptible. Consequently, these observations revealed the relevance of sampling inflorescences from plants grown and producing under controlled conditions. From this point of view, our optimized agronomic technique leading to the production of flowering cuttings in 3 months turned out to be crucial in grapevine, since it is a fruit crop species with long reproducing cycle. An alternative to flowering/fruiting cutting production is represented by the use of microvine, a dwarf stature, short generation cycle and continuous flowering genotype derived from the pure L1 of Pinot meunier (Boss and Thomas, 2002). Anyway, the microvine needs to be crossed with the desired genotype to transmit it the early and continuous flowering phenotype; this step can encounter inter-fertility problems and is time-consuming with the aim to assess disease resistance at inflorescence level.

2.4.3. Phenotype predictability based on a single organ evaluation

Organ A in vivo vs organ B in vivo. At all three studied phenological stages, we found general significant positive correlations between leaf and inflorescence organ *in vivo.* Anyway, interestingly, an organ distinctiveness in DM response emerged in the clear case of Cabernet Cortis, along with few cases presenting subtle phenotype differences. This can be considered a case of divergent dual epidemics, which are described as infections differently developing on two or more plant organs during a growing season. Agricultural pathosystems where they occur are often relevant, because the harvestable and valuable part corresponds to one of the affected organs (Savary et al., 2009). In terms of harvestable organs, grapevine is emblematic given the relevance of grape itself and as a number of derived commodities and transformed products, among which wine is the noblest one. Unlike the single leaf organ of various *Vitis* genetic backgrounds (e.g. Gobbin et al., 2003; Koopman et al., 2007; Rouxel et al., 2012), characterization studies on pathogenic races derived from different organs within the same genotype have not been performed yet. Therefore, we cannot exclude this possibility to explain the found divergent dual epidemics

in Cabernet Cortis. In regards to this, the resistance genetic nature is of paramount importance: vertical resistance (qualitative, specific resistance, or hypersensitivity) is usually monogenic and effective only for a subset of pathogen races, while horizontal resistance (quantitative, non-specific, or general resistance) is partial or polygenic and thought to be effective against all races of the pathogen (da S Pereira et al., 2012). Cases of divergence in intra-genotype DM response have been phenotypically evaluated in field. For instance, Basler and Pfenninger (2003) found high resistance to Johanniter clusters and low resistance on leaves. Further, Kennely et al. (2005) observed that the inter-specific hybrid cv. Chancellor had highly susceptible fruits, but foliage was nearly immune; by contrast, the inter-specific hybrid cv. Delaware had highly susceptible foliage, but fruits were rarely infected. Lately, Savary et al. (2009) also reported on a non-linear modelling and logistic regression indicating non-linearity in the *in vivo* foliage–cluster relationships in the case of grapevine DM. This aspect is crucial in grapevine breeding to combine foliage and cluster resistance.

Organ A in vitro vs organ B in vivo. Our findings highlighted that *in vitro* leaf disc bioassay was not predictive (no significant correlation) of the *in vivo* inflorescence/bunch phenotype. Although barely comparable, given the different statistical approach, this result is in agreement with Calonnec et al. (2013), who reported on the reliability of grapevine leaf bioassays for predicting DM resistance on fruit in the field only above the threshold of OIV 452-1 = 5. In fact, our sample set encompasses genotypes with the corresponding Dis parameter ranging in average from 3.50 to 8.75 at leaf disc level. Moreover, it should be considered that different DM resistance-related (R) loci (*Rpv1* and *Rpv3*) were present in the genetic background of their studied *Muscadinia rotundifolia* and Regent-derived genotypes, compared to the various R loci (*Rpv3, Rpv10, Rpv12*) inherited in our studied genotype pool (VIVC, 2015; Vezzulli S. pers. comm.).

Organ A in vitro vs organ B in vitro. The new *in vitro* phenotyping method developed to evaluate DM response on inflorescences allowed us to compare *in vitro* assessments of both leaf and inflorescence DM response. Focussing on the E-L 17 phenological stage, we found a general significantly positive correlation comparing the field-derived leaf Int results with both inflorescence Dis and Sd. By contrast, the AUDPC leaf values did not significantly correlate with the inflorescence spore counts; this is expected since, as quantitative parameters, they evaluate different aspects. AUDPC reflects the progress of the pathogen sporulation in time, while the spore count is the number of sporangia in a precise moment post-infection. Looking at the resulted phenotypes in depth, Cabernet Cortis showed MR

leaves and MS inflorescences. The latter represents an interesting result, confirmed by organs derived from controlled conditions as well, and corroborates the diverse DM epidemiological behaviour at organ level previously observed *in vivo,* making Cabernet Cortis a valuable model to study divergent dual epidemics.

To date, several transcriptomics studies on DM response have been reported at foliar level (e.g. Polesani et al., 2010; Perazzolli et al., 2012; Li et al., 2015), but no research works have been reported to dissect the disease response at inflorescence/bunch level. In conclusion, our optimized and developed phenotyping strategies are of practical interest not only for breeding applications, but also preparatory to genetics-genomics, transcriptomics and metabolomics studies.

2.5. ACKNOWLEDGEMENTS

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2.6. APPENDIX 1

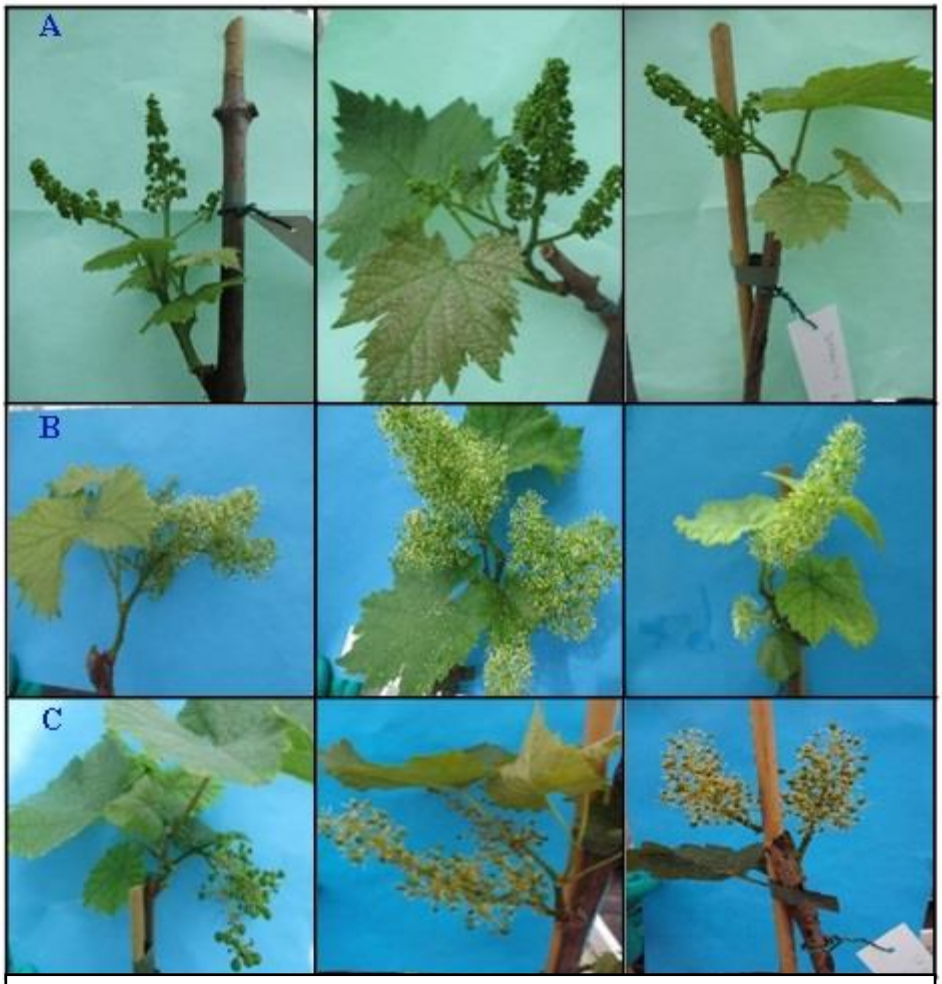

Fig. S2.1. Fruiting cutting production. Panel A: the studied E-L 17 stage, Panels B: the studied E-L 25 stage, Panel C: the studied E-L 29 stage.

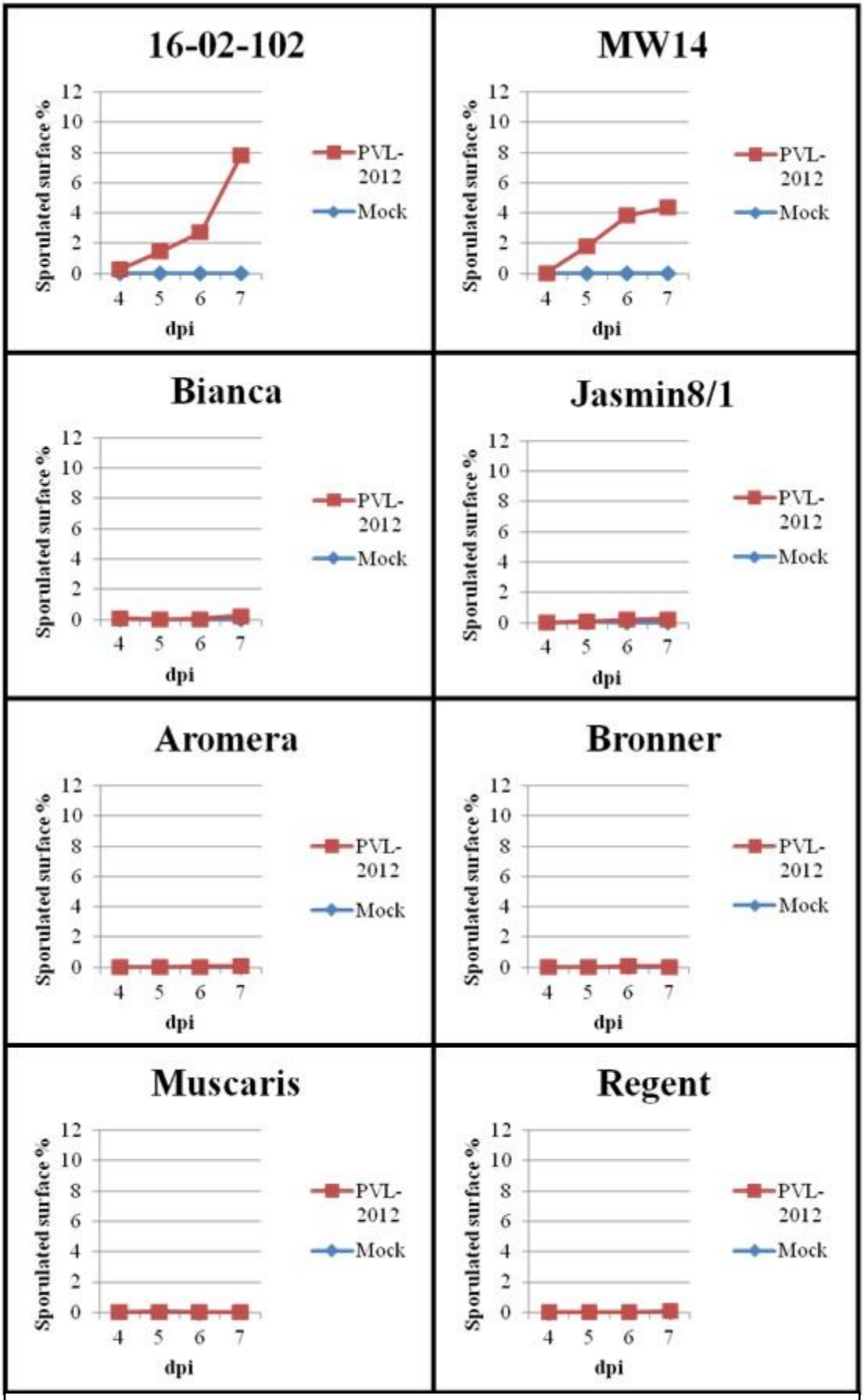

Fig. S2.2. Percentage of leaf disc sporulated surface on eight *Vitis* hybrids upon downy mildew (PVL-2012) infection versus mock treatment, at different days post-infection (dpi).

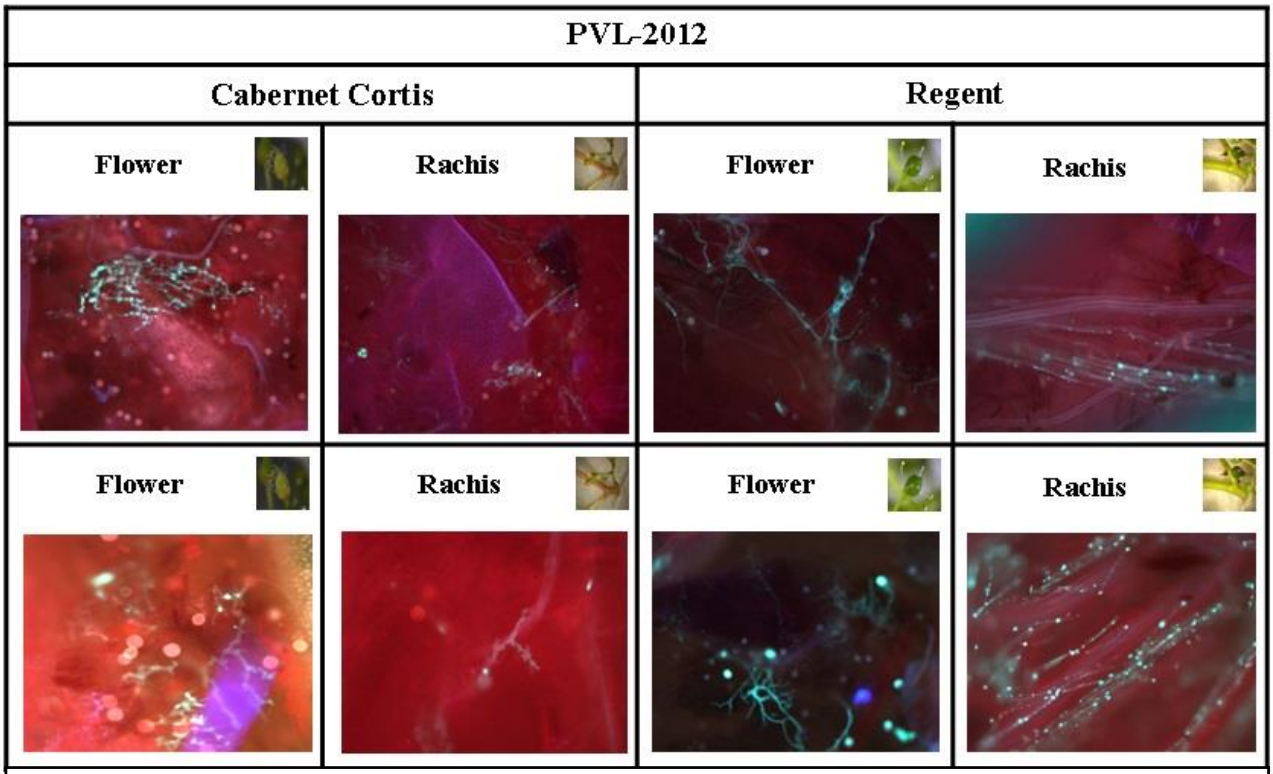

Fig. S2.3. Extended study staining results on flower and rachis of the mid-susceptible Cabernet Cortis and the mid-resistant Regent upon downy mildew (PVL-2012) infection at 6 dpi (top picture 5X zoom; bottom picture 20X zoom).

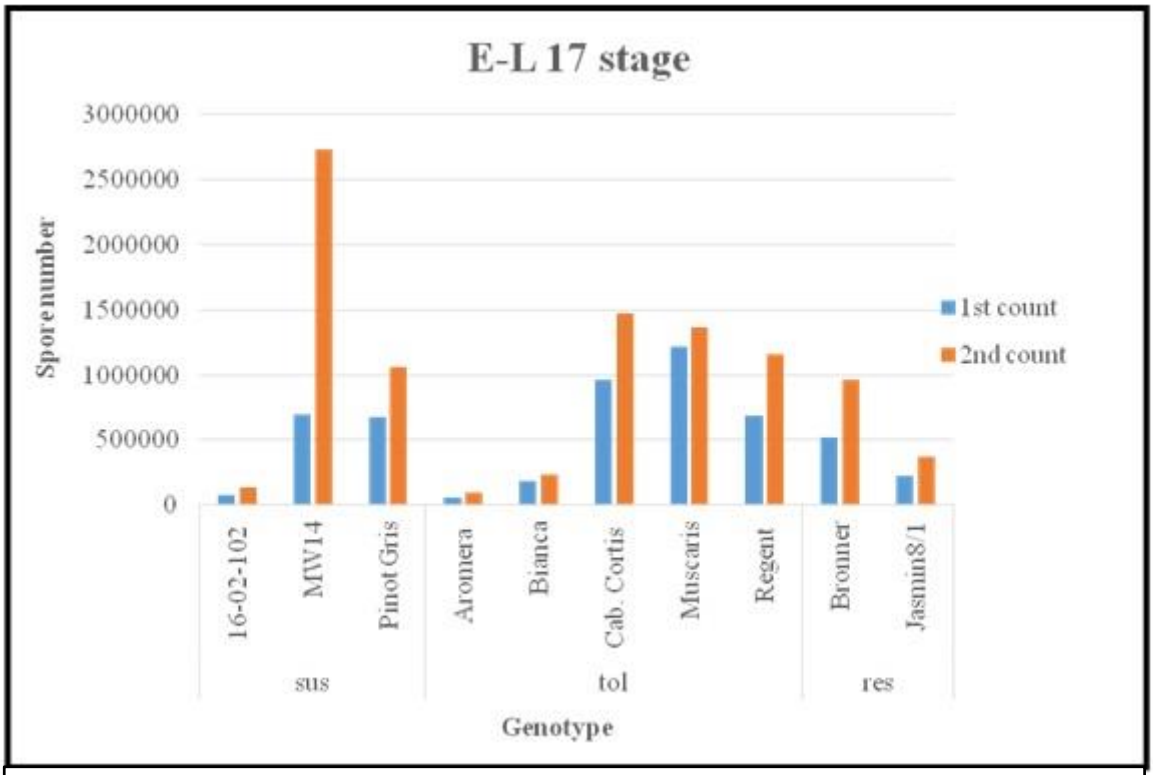

Fig. S2.4. Spore quantification on inflorescences upon downy mildew infection at the most reactive E-L 17 phenological stage.

Table S2.1. Scores of the three evaluated parameters on field- and/or phytotron-derived leaf and inflorescence of the 11 studied genotypes. dpi: days post-infection, Dis: disease impact, Sd: sporulation density, and Int: integrated index.

			Field-derived			Phytotron-derived		
Genotype	**Organ (detail)**	**dpi**	**Mean Dis**	**Mean Sd**	**Int**	**Mean Dis**	**Mean Sd**	**Int**
16-02-102	leaf (disc)	4	6.83	7.33	7.08	-	-	-
MW14	leaf (disc)	4	8.08	8.42	8.25	-	-	-
Pinot Gris or Noir	leaf (disc)	4	7.58	7.58	7.58	5.29	6.21	5.73
Aromera	leaf (disc)	4	9	9	9	-	-	-
Bianca	leaf (disc)	4	8.42	8.42	8.42	-	-	-
Bronner	leaf (disc)	4	9	9	9	-	-	-
Cabernet Cortis	leaf (disc)	4	8.75	8.75	8.75	6.57	7.21	6.89
Jasmin8/1	leaf (disc)	4	8.67	8.58	8.62	-	-	-
Muscaris	leaf (disc)	4	8.75	8.75	8.75	-	-	-
Regent	leaf (disc)	4	9	9	9	-	-	-
16-02-102	leaf (disc)	7	3.50	4.67	4.04	-	-	-
MW14	leaf (disc)	7	4.25	5.37	4.75	-	-	-
Pinot Gris or Noir	leaf (disc)	7	4	3.50	3.74	3.57	4.36	3.94
Aromera	leaf (disc)	7	7.58	7.67	7.62	-	-	-
Bianca	leaf (disc)	7	6.83	6.83	6.83	-	-	-
Bronner	leaf (disc)	7	8.75	8.75	8.75	-	-	-
Cabernet Cortis	leaf (disc)	7	6.92	6.25	6.57	4.71	5.64	5.16
Jasmin8/1	leaf (disc)	7	6.50	6.25	6.37	-	-	-
Muscaris	leaf (disc)	7	7.58	7.75	7.67	-	-	-
Regent	leaf (disc)	7	8.67	8.67	8.67	-	-	-
16-02-102	inflorescence (E-L 17)	6	5	5	5	-	-	-
MW14	inflorescence (E-L 17)	6	5	7	5.92	-	-	-
Pinot Gris or Noir	inflorescence (E-L 17)	6	6.20	6.20	6.20	1	5	2.24
Aromera	inflorescence (E-L 17)	6	9	9	9	-	-	-
Bianca	inflorescence (E-L 17)	6	7	7	7	-	-	-
Bronner	inflorescence (E-L 17)	6	9	9	9	-	-	-
Cabernet Cortis	inflorescence (E-L 17)	6	4.33	5.89	4.85	5.67	3	4.12
Jasmin8/1	inflorescence (E-L 17)	6	9	9	9	-	-	-
Muscaris	inflorescence (E-L 17)	6	9	9	9	-	-	-
Regent	inflorescence (E-L 17)	6	7	7	7	-	-	-

Table S2.2. Different combinations between soaking time and *P. viticola* spore concentration performed in the pilot study. x: satisfying assays, and X: best combination.

	Soaking time		
PVL-2012 sporangia ml^{-1}	1h	2h	3h
0 (mock)	–	–	–
5,000	–	–	–
10,000	–	**X**	x
20,000	x	x	–

Table S2.3. Means of integrated index (Int) scores on leaf and inflorescence with their standard errors (SE).

Genotype	**Mean Leaf Int**	**SE Leaf Int**	**Mean Inflorescence Int**	**SE Inflorescence Int**
16-02-102	4.0	0.05	5	0
MW14	4.7	0.08	5.9	0
Pinot Gris	3.6	0.06	6.2	0.2
Aromera	7.6	0.04	9	0
Bianca	6.8	0.06	7	0
Bronner	8.8	0.03	9	0
Cabernet Cortis	6.5	0.06	4.8	0.2
Jasmin8/1	6.3	0.06	9	0
Muscaris	7.6	0.05	9	0
Regent	8.7	0.03	7	0

Table S2.4. Comparisons between the evaluated integrated index on field- and phytotron-derived organs of genotypes of interest. Panel A: Cabernet Cortis. Panel B: Pinot Noir (— = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, / = not calculated).

A				
Genotype			Cabernet Cortis	
	Origin		Phytotron	
		Organ	Leaf (disc) (Infected)	Inflorescence (Infected)
Cabernet Cortis	Field	Leaf (disc) (Infected)	—	/
		Inflorescence (Infected)	/	—
B				
Genotype			Pinot (Noir)	
	Origin		Phytotron	
		Organ	Leaf (disc) (Infected)	Inflorescence (Infected)
Pinot (Gris)	Field	Leaf (disc) (Infected)	—	/
		Inflorescence (Infected)	/	**

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CHAPTER 3

Grapevine downy mildew dual epidemics: a leaf and inflorescence transcriptomics study

Abstract

Downy mildew (DM), caused by biotrophic oomycete *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, is one of the most important plagues affecting viticulture, especially in temperate-humid climates. We have previously identified in the *Vitis* hybrid Cabernet Cortis a model to study divergent dual epidemics in DM since displays mid-resistant leaves and mid-susceptible inflorescences. The changes induced by *P. viticola* were investigated by combining phenotypical, histological, ultrastructural and transcriptomical approaches, in order to provide comprehensive information about the different organ-response. To identify potential organ- and genotype-dependent transcriptional responses and functions associated with the different levels of resistance/susceptibility, we sequenced and analyzed the transcriptomes of the hybrid Cabernet Cortis and one of its parents *V. vinifera* Cabernet Sauvignon at 48 hours post inoculation. Transcriptomes were compared to identify constitutive differences and DM-inducible responses that may underlie their different phenotypes. Responses to *P. viticola* in mid-resistant Cabernet Cortis leaf were characterized by an up-regulation of genes related to secondary metabolisms, particularly the stilbene synthases. Genes implicated in the secondary metabolism, response to stimuli, protein modifications, carbohydrate metabolism and reproduction are up-regulated in mid-susceptible inflorescences of Cabernet Cortis, conversely these genes are poorly transcribed or down-regulated in susceptible inflorescences of Cabernet Sauvignon. This study provides a first exploration of the functions associated with varying levels of resistance/susceptibility to DM in the *Vitis* hybrid Cabernet Cortis that can shad light into the molecular basis underpinning divergent DM dual epidemics.

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"Grapevine downy mildew dual epidemics: a leaf and inflorescence transcriptomics study"

3.1. INTRODUCTION

In agricultural settings, dual epidemics are pathogen infections that develop on two or more plant organs in the course of a cropping season. Dual epidemics are particularly important when one of the organs affected by the disease constitutes the harvestable part of the plant (Savary et al., 2009). In grape production for example, lesions on leaves result in physiological injuries such as a reduction of photosynthetic activity, while infections of the berries lead to decreased yield and fruit quality with important economic losses. Dual epidemics are known in several pathosystems with important economic implications, including apple scab (Holb et al., 2005), strawberry powdery mildew (Carisse et al., 2013), and rice blast (Ghatak et al., 2013).

Grapevines are host of a variety of economically important pathogens, some of which differentially infect the vegetative and reproductive organs. In viticulture, dual epidemics were described for grey mould (Vatsa-Portugal et al., 2015), downy mildew (DM) (Boso et al., 2011, Kennelly et al., 2005, Calonnec et al., 2013), powdery mildew (Calonnec et al., 2013), and *Phylloxera* (Winkler et al*.,* 1974). Grapevine dual epidemics are often complex to manage, because the association between epidemiological components occurring on different organs has been scarcely investigated, and because to foresee the risk toward the harvestable grapes has been barely attempted.

Grapevine DM, caused by *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, is recognized as one of the most economically important pathogens of grapes in many wine grape growing regions (Gindro et al., 2012). All but one cultivated varieties of *Vitis vinifera* L. are highly susceptible to *P. viticola* under environmental favourable conditions. DM is particularly aggressive in temperate-humid climates with warm and wet weather during the growing season (Musetti et al., 2006). To control the disease, frequent applications of chemical fungicides are required resulting in environmental damage and human health hazard. *P. viticola* is an obligate biotrophic pathogen capable of infecting all green tissues of the grapevine (leaves, inflorescences and tendrils). With mean temperature above 10°C and rain precipitations, macrosporangia release bi-flagellated cells called “zoospores”. Swimming on wet organ surfaces, zoospores reach the functional stoma where they encyst, develop a germ tube and penetrate the substomatal cavity with the primary hypha (Unger et al., 2007, Lenzi et al., 2015). The parasite creates intimate contact with the host cells, degrades the cell wall and shapes a specialized intracellular structure called haustorium, causing the invagination of the plasma membrane (Unger et al., 2007). Typical symptoms on the adaxial surface are called “oil spots”, because they appear as yellow spots with an

oily appearance (Musetti et al., 2006). On the abaxial surface, white mould appears due to fungal sporulation; it also appears on the young inflorescences and tendrils.

At the foliar level, mechanisms of resistance to DM have been characterized both from a histological and a transcriptional point of view. In the incompatible interactions, the response of resistant genotypes is a post-infection phenomenon (Polesani et al., 2010), following the haustorium formation (Díez-Navajas et al., 2008, Liu et al., 2014), marked by strong and rapid transcriptional reprogramming (Polesani et al., 2010, Figueiredo et al., 2012). Grapevines can activate a broad spectrum of defence mechanisms in response to *P. viticola* infection as formation of callose in the stomata (Gindro et al., 2003, Yu et al., 2012), lignification process and production of reactive oxygen species (ROS) (Kortekamp, 2006), induced peroxidases and the hypersensitive reaction (HR) (Díez-Navajas et al., 2008). On one hand callose deposition around the stomata limits nutrient exchange and prevent secondary infections (Toffolatti et al., 2012); on the other, localized generation of ROS, especially of hydrogen peroxide (H_2O_2), has been related to signal transduction, cell-wall reinforcement, hypersensitive response (HR) and phytoalexin production (Malacarne et al., 2011, Yu et al., 2012, Liu et al., 2014). Expression studies reveal the induction of genes involved in the defense response, such as the synthesis of pathogenesis-related (PR) proteins (Kortekamp, 2006, Polesani et al., 2010, Malacarne et al., 2011, Lenzi et al., 2015) – particularly β-glucanase and chitinase (Perazzolli et al., 2011) – and in the transcription regulation, with a rapid induction of the transcription factor WRKY33 (Merz et al., 2015). Furthermore, the presence of secondary metabolites such as phytoalexins or antimicrobial compounds have been largely characterized (Pezet et al., 2004, Alonso-Villaverde et al., 2011, Ali et al., 2012). Among phytoalexins, resveratrol – a low molecular weight stilbene – and its oxidation products are synthesized in response to both biotic (e.g. fungal infection) as well as abiotic stresses (e.g. UV radiation) (Langcake & Pryce, 1977, Chong et al., 2009, Jeandet et al., 2010).

In the susceptible genotypes there is a compatible interaction (Polesani et al., 2008, Perazzolli et al., 2012) and an abortive defence response during the early steps of infection (Polesani et al., 2010). Most of the differentially expressed genes (DEGs) were down-regulated, particularly genes related to photosynthesis and carbon metabolism (Polesani et al., 2008, Gamm et al., 2011). Moreover, the up-regulation of genes involved in defence, response to stimuli and phenylpropanoid pathway is insufficient (Polesani et al., 2008, Perazzolli et al., 2012).

To date, the transcriptional studies in grapevine upon *P. viticola* inoculation were focused on RNA extracted from leaves (Kortekamp, 2006, Polesani et al., 2008, Polesani et al., 2010, Malacarne et al., 2011, Perazzolli et al., 2012, Lenzi et al., 2015, Merz et al., 2015). Transcriptional changes occur in all the infected organs, but little is known about gene regulation during DM-grapevine interaction in different tissues.

The aim of the present work is to understand the molecular basis underpinning divergent DM dual epidemics in two different grapevine organs, leaf and inflorescence. The different response between organs of two *Vitis* hybrids and two *V. vinifera* varieties were studied at phenotypical, histological and ultrastructural level. Furthermore, the organ transcriptomes of one hybrid and one variety were analyzed in order to identify DEGs at baseline and upon *P. viticola* infection.

3.2 MATERIALS AND METHODS

3.2.1. Biological materials

Based on a previous phenotypic screening under field and controlled conditions (Buonassisi et al., submitted), we selected the *Vitis* hybrid Cabernet Cortis (CC) and one of its parents *V. vinifera* cv. Cabernet Sauvignon (CS), which display different susceptibility to DM. The former has mid-resistant (MR) leaves and mid-susceptible (MS) inflorescences, while the latter has both susceptible (S) leaves and inflorescences (Fig. S3.1). In addition, a reference *V. vinifera* variety – Pinot Noir (PN) – and a CC-related *Vitis* hybrid – Muscaris (Mus) – were selected for validation of RNA-Seq analysis (Table 3.1).

Fruiting cuttings, namely cuttings which produce 4 leaves and an inflorescence – Eichhorn-Lorenz (E-L) scale 17 (Eichhorn and Lorenz, 1977) – within 3 months from planting, were obtained using a protocol we optimized (Buonassisi et al., submitted).

Table 3.1. The 4 studied grapevine genotypes, followed by their pedigree, taxon, releasing country, FEM origin and information on downy mildew organ response level.

Genotype (clone)	Pedigree	Taxon	Releasing country	FEM origin	DM response level	
					Leaf	Inflorescence
Cabernet Cortis	Cabernet Sauvignon x Solaris	*Vitis* hybrid	Germany	Phytotron	mid-resistant (MR)	mid-susceptible (MS)
Cabernet Sauvignon	Cabernet Franc x Sauvignon Blanc	*Vitis vinifera*	France	Phytotron	susceptible (S)	susceptible (S)
Muscaris	Moscato Bianco x Solaris	*Vitis* hybrid	Germany	Phytotron	mid-resistant (MR)	mid-resistant (MR)
Pinot Noir (ENTAV115)	-	*Vitis vinifera*	France	Phytotron	susceptible (S)	susceptible (S)

3.2.2. Downy mildew *in vivo* inoculation and symptom assessment

P. viticola inoculum, named PVL-2014, was collected from *V. vinifera* plants of cv. Chardonnay in an untreated field in the Trentino province (north-eastern Italy). Sporangiophores were vacuumed, collected in filtered tips, stored in 15mL falcon tubes and frozen. Two weeks before the inoculation, sporangiophores were propagated and maintained by subsequent inoculations on PN leaves. *P. viticola* sporangia were collected on the abaxial surfaces which bore freshly sporulating lesions. The inoculum concentration was adjusted to 10^5 sporangia mL^{-1} with Malassez's counting chamber (Perazzolli et al., 2012).

Six fruiting cuttings of each genotype were divided into two equal groups for mock- and *P. viticola*-inoculation. The leaves and the inflorescence of three biological replicates were sprayed with *P. viticola* inoculum suspension, while the remaining three were mock-inoculated (distilled water). Two hours before the inoculation, the leaves and inflorescences were moistened with cold, distilled water to simulate natural rainfall. After inoculation all cuttings were incubated overnight in the dark at 25°C with 99–100% RH and then kept under controlled conditions: temperature at 27°/22°C (day/night), photoperiod 16/8h (light/dark) and 90% RH to allow the development of *P. viticola*. To promote the sporulation, six days after inoculation with the pathogen, all cuttings were incubated overnight in the dark at 25°C with 99–100% RH. The spread of the disease was visually assessed at 7 days post-inoculation (dpi) on leaves and inflorescences based respectively on the OIV 452 and OIV

453 descriptors (OIV, 2009). Significant differences in disease symptoms between conditions were detected via a parametric t-test ($p < 0.05$).

3.2.3. Detection of H_2O_2 in inoculated organs using DAB Staining

The inoculated leaves and inflorescences were sampled at 7 dpi and observed using a stereomicroscope (Leica MZ16F). Afterwards they were rinsed in distilled water and immersed in a solution containing 1 mg/ml 3,3-diaminobenzidine (DAB, Sigma) dissolved in HCl-acidified (pH 3.8) distilled water (Thordal-Christensen et al., 1997). Samples were incubated for 5 h to allow the uptake and polymerization of DAB and the reaction with H_2O_2 and peroxidase. The leaves were observed with the abaxial surface uppermost while the flower clusters were analysed focussing on a single inflorescence. H_2O_2 accumulation in the leaf or inflorescence tissues was visualized as reddish-brown spots.

3.2.4. Transmission electron microscopy

Leaf and inflorescence samples were collected at 7 dpi by fruiting cuttings and used, half part for microscopy and half for RNAseq analyses.

Ultrastructural interactions between plant and pathogen on the different tissues were studied by a transmission electron microscope (TEM). Small samples were dissected from the infected leaves (1 × 3 mm) and inflorescences (1 × 3 × 2 mm) as well as from the mock-inoculated samples. Thereafter samples were fixed in 3% glutaraldehyde, rinsed in buffer, postfixed in 1% osmium tetroxide in 0.1 M potassium phosphate for 2 h at 4°C, dehydrated in ethanol, and embedded in Epon-Araldite resin according to the method described by Musetti et al. (2006). Several serial ultrathin sections (60-70 nm in thickness) were cut using an ultramicrotome (Reicher Leica Ultracut E ultramicrotome, Leica Microsystems, Wetzlar, Germany) and collected on 200 mesh uncoated copper grids. Sections were then stained with uranyl acetate and lead citrate and observed under a PHILIPS CM 10 (FEI, Eindhoven, The Netherlands) TEM, operated at 80 kV.

3.2.5. Sample collection and RNA isolation

Leaf and inflorescence samples were collected from mock- and *P. viticola*- inoculated plants at 48 hours post-inoculation (hpi). This time point was chosen because in susceptible genotypes it was associated with leaf colonization by primary hyphae; whereas their growth is retarded and rarely completed in the resistant genotypes (Unger et al., 2007, Liu et al.,

2014). Furthermore, Polesani et al. (2010) measured a sharp increase in the levels of both jasmonic acid (JA) and methyl jasmonate (MeJA) in resistant *V. riparia* leaves at 48 hpi. Eight conditions were analysed by RNA-Seq: CC leaf control (CCLC), CC leaf *P. viticola*-inoculated (CCLI), CC inflorescence control (CCIC), CC inflorescence *P. viticola*-inoculated (CCII), CS leaf control (CSLC), CS leaf *P. viticola*-inoculated (CSLI), CS inflorescence control (CSIC) and CS inflorescence *P. viticola*-inoculated (CSII). For each condition, leaf and inflorescence samples from three replicates (fruiting cuttings) were collected and each sample comprised two leaves and half inflorescence taken from the same plant. Samples were immediately frozen in liquid N_2 and stored at −80°C. Total RNA was extracted using the Spectrum Plant total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) and quantified using a NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA integrity was confirmed by 1% agarose gel electrophoresis and using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

3.2.6. Library construction and mRNA sequencing

To analyse the genome-wide transcriptional responses to DM, twenty-four RNA-seq libraries were constructed. The poly(A) mRNA was isolated by 800 ng of total RNA using a NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs, Massachusetts, USA) and library was constructed with the NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs, Massachusetts, USA) according manufacturer's instructions. The library quality was assessed by the Agilent 2100 Bioanalyzer and sequencing was performed on a NextSeq 500 at the Functional Genomic Center, University of Verona (ddlab.sci.univr.it/fgl). All libraries were individually barcoded in order to allow the multiplexing of libraries in a single chip. Fragments were sequenced as 75 nucleotide (nt) single-end reads.

3.2.7. Read processing

Adapter trimming was carried out with Scythe v.0.991 (https://github.com/ucdavis-bioinformatics/scythe) with a prior of 0.4. Quality trimming and filtering of the raw reads was carried out with Sickle v.1.21 (https://github.com/ucdavis-bioinformatics/sickle); reads with a Phred quality score lower than 20 and shorter than 50 nt were then discarded. The *V. vinifera*

line ‘PN40024’ transcriptome (version V1 from http://genomes.cribi.unipd.it/grape/; Jaillon et al., 2007) and a non-redundant dataset of 99 *P. viticola* cDNA sequences (Table S3.1) were combined and used as a reference for reads mapping. The *P. viticola* cDNA sequences codifying for membrane, citoplasmatic and nuclear proteins, and trascription factors were selected. Single-end reads were mapped using Bowtie2 v.2.2.3 (Langmead & Salzberg, 2012), with the following parameters: -q –end-to-end –sensitive –no-unal -p 12 -U. Read counts for each grapevine and *P. viticola* gene were obtained with the program sam2counts.py v.0.91 (https://github.com/vsbuffalo/sam2counts). Uniquely mapped reads were used to assess expression levels of grapevine and *P. viticola* genes.

3.2.8. Identification and functional annotation of differentially expressed genes

Read counts were normalized using the Bioconductor package DESeq2 v.2.1.2.10. (Love et al., 2014). A false discovery rate (FDR) of Benjamini-Hochberg multiple tests lower than 5 % (*Padj* < 0.05) with a minimum Log2 fold change of 1.0 were imposed to identify DEGs through pairwise comparison. Eight pairwise comparisons between the organs of the studied genotypes were analysed: CCLC vs. CSLC, CCIC vs. CSIC, CCLC vs. CCIC, CSLC vs. CSIC, CCLI vs. CCLC, CCII vs. CCIC, CSLI vs. CSLC and CSII vs. CSIC. In total, 16,322 grapevine DEGs were classified into functional categories of Gene Ontology (GO) biological process terms using the annotations provided in VitisNet (Grimplet et al., 2009). Enrichment analyses of grapevine biological functions were computed using the hypergeometric test (Pearson's exact test) implemented in the Biological Networks Gene Ontology (BiNGO) software (Maere et al., 2005). A cut-off of $P \leq 0.05$ was set to determine statistical significance. Gene ontology enrichment networks were visualized with Cytoscape version 3.2.1 (Shannon et al., 2003). Redundancies were removed and significantly overrepresented ($P < 0.05$) GO biological process terms were identified for the up- and down-regulated genes of each cluster.

3.2.9. Primers design analysis

Among grapevine DEGs, some candidate genes were selected (Table S3.2), their sequences and their respective isoforms were aligned using the online T-Coffee Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/tcoffee/) program to select specific nucleotide regions allowing the unambiguously gene target amplification. “OLIGO Primer Analysis” (http://www.oligo.net/) software was used to evaluate Tm (melting temperature), CG content, self-annealing and loop parameters of the specific and manually designed primers.

3.3. RESULTS AND DISCUSSION

3.3.1. Phenotypical and histological data reveal different organ-response upon *P. viticola* inoculation

3.3.1.1. *In vivo* disease assessment

To assess the *P. viticola* symptoms at 7 dpi, the OIV 452 and OIV 453 descriptors were used for leaves and inflorescences, respectively. In detail, *Organisation Internationale de la Vigne et du Vin* (OIV) code refer to a discrete scale based on five classes (1, 3, 5, 7 and 9) ranging from the most susceptible (1, extended sporulation) to the totally resistant (9, no symptoms at all) genotype (OIV, 2009). To standardize the DM symptom assessment in addition to the mentioned OIV code, two international codes can be used: the European and Mediterranean Plant Protection Organization (OEPP/EPPO, 2001) and the International Union for the Protection of New Varieties of Plants (UPOV, www.upov.int). However the OIV descriptors are the most widely deployed, in particular OIV 452 and OIV 453 are related to in field and *in/ex vivo* DM symptom evaluation on leaves and inflorescence/clusters.

Upon *P. viticola* inoculation, at 7 dpi CC, carrying *Rpv 3* and *Rpv 10*, presented MR leaves showing a mean OIV 452 score of 5.7, while the inflorescence was classified as MS displaying an OIV 453 score of 3.6 (Fig. 3.1). CS organs resulted S to *P. viticola*, presenting a mean value of 2.5 and 2.3, respectively (Fig. 3.1). Mock-inoculated organs had the same behaviour presenting a score 9 for both genotypes. Significant differences in disease symptoms between pairwise conditions were detected via the parametric t-test ($P < 0.05$), using the mean OIV scores. Comparing the two inoculated organs within the same genotype, CC presented significant phenotypical differences ($P < 0.05$) due to the different organ resistance/susceptibility, while CS did not show a significant response variation upon pathogen attack ($P = 0.84$) owing to the same organ behaviour (Table 3.2A). Comparing the same organ between CC and CS, at foliar level both genotypes showed a significantly different response to *P. viticola* inoculation ($P < 0.01$), resulting to be respectively MR and S. Regarding inflorescences, they did not show significant differences ($P = 0.40$), as result of the similar OIV 453 values ascribing them to the MS and S classes, respectively (Table 3.2B). Significant differences in the *in vivo* disease assessment sustain the reliability in the use of fruiting cuttings under controlled conditions to study grapevine dual epidemics, to date phenotypically evaluated only in field (Basler and Pfenninger, 2003, Kennelly et al., 2005, Savary et al., 2009).

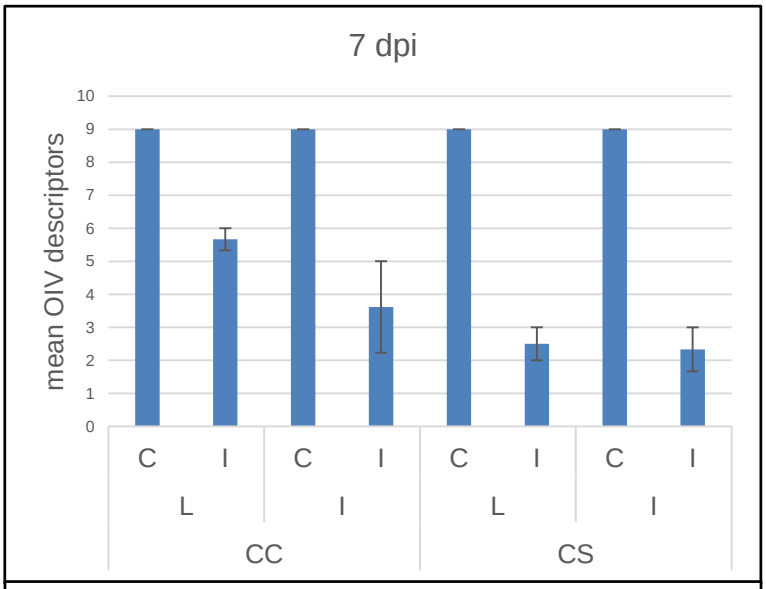


Fig. 3.1. Means of OIV452/453 descriptors scores of Cabernet Cortis (CC) and Cabernet Sauvignon (CS) respectively on leaf (L) and inflorescence (I) at 7 days post infection (dpi). The histogram reports the mean scores of mock- (C) and *P. viticola*-inoculated (I) samples.

Table 3.2. Comparisons between the mean OIV452/453 scores on leaf and inflorescence of Cabernet Cortis and Cabernet Sauvignon. Panel A: comparison between the different organs within the same genotype. Panel B: comparison of the same organ between the genotypes of interest. (— = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, / = not calculated).

A			
	Organ		
	Leaf (Inoculated vs Mock)	Inflorescence (Inoculated vs Mock)	Leaf-Inflorescence (Inoculated vs Inoculated)
	Parameter	**Parameter**	**Parameter**
Genotype	mean OIV452	mean OIV453	mean OIV452/ mean OIV453
Cabernet Cortis	***	*	*
Cabernet Sauvignon	***	**	—

B				
Genotype			Cabernet Sauvignon	
	Organ		Leaf (Inoculated)	Inflorescence (Inoculated)
		Parameter	mean OIV452	mean OIV453
Cabernet Cortis	Leaf (Inoculated)	mean OIV452	**	/
	Inflorescence (Inoculated)	mean OIV453	/	—

3.3.1.2. Analysis of *P. viticola* development and of the associated H_2O_2 accumulation

To assess the response of different grapevine organs to *P. viticola* attack, disease development was assessed looking at the pathogen development under a stereomicroscope. The leaves of CC showed some limited and irregular attack areas with sparse sporulation and necrotic spots (red arrows in Fig. 3.2B); on the other hand, on the inflorescence abundant and localized sporulation was visible (Fig. 3.2B). The organs of CS were characterized by abundant and widespread sporulation both on the leaf surface and on the inflorescence (Fig. 3.2B). Since H_2O_2 interferes with pathogen growth and diffusion (Yu et al., 2012, Liu et al., 2014), we histochemically analysed its accumulation at 7 dpi. No DAB staining was detected in foliar and inflorescence tissues from all mock-inoculated samples (Fig. 3.2C). H_2O_2 accumulation was clearly visible on the surface of CC leaves (red arrows in Fig. 3.2D). H_2O_2 accumulation was not detectable on the DM infected CC inflorescence and on the CS leaf and flowers (Fig. 3.2D). Altogether, macroscopical and histological response to DM showed that in the mid-resistant leaf the sporulation was related to the H_2O_2 production, while in the mid-susceptible or susceptible organs they were not related each other. These results were in agreement with the histological survey undertaken by Liu et al. (2014), which analised the sporulation, callose deposition and H_2O_2 production on the leaves of three resistant Chinese wild grapevines and a susceptible European cultivated variety.

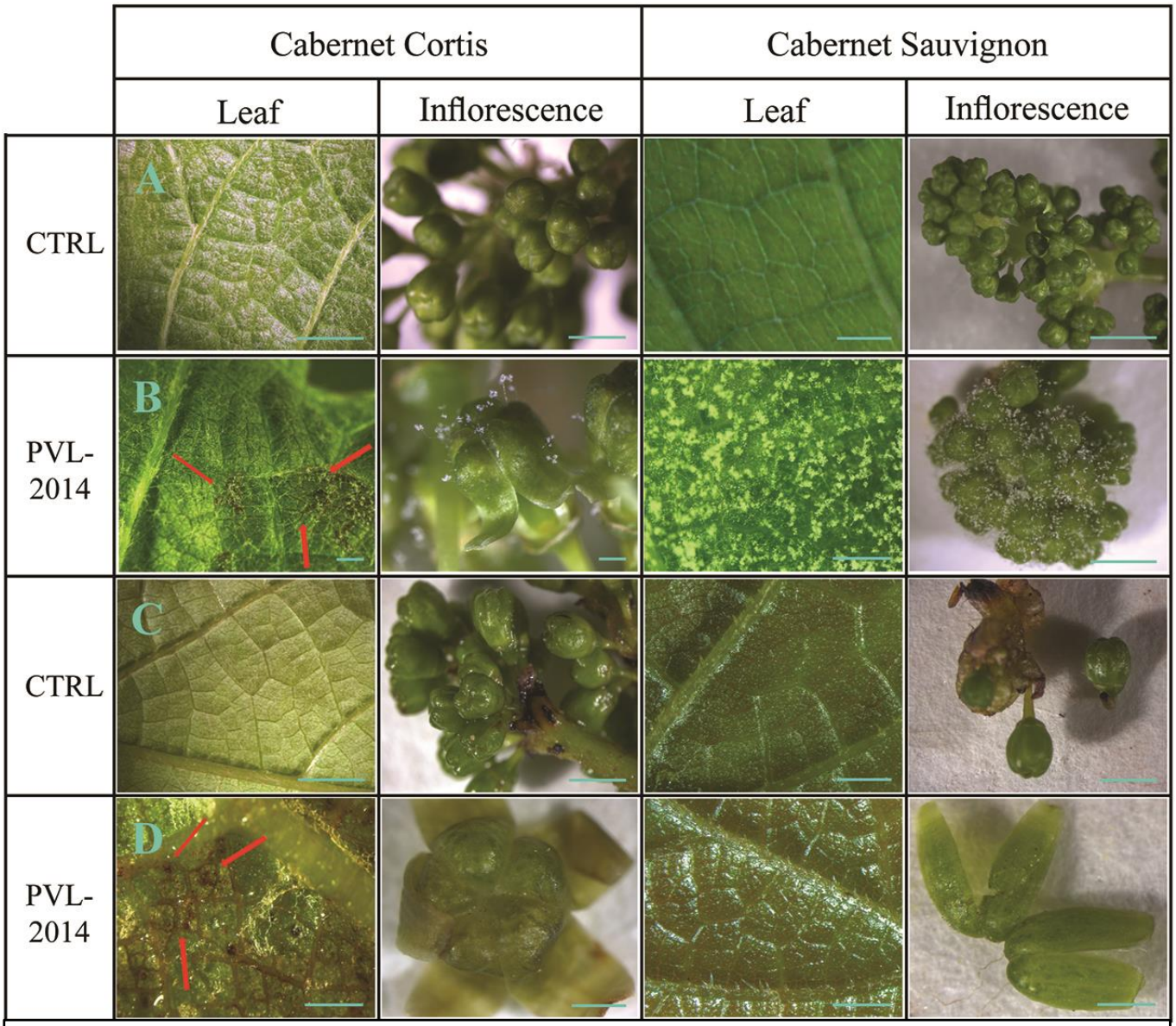


Fig. 3.2. Stereomicroscopy imaging of control (panels A and C) and *P. viticola*-inoculated (panels B and D) grapevine leaves and inflorescences.
Panel A: in Cabernet Cortis and Cabernet Sauvignon control samples no sporulation or tissue modifications are visible. Panel B: inoculated tissues of Cabernet Cortis show limited sporulation and necrotic dots (red arrows) on the leaves; on the inflorescences, sporulation is visible; on the contrary, necrotic areas are absent. A widespread sporulation is detectable on Cabernet Sauvignon leaf and inflorescence.
Panel C: no DAB staining is observed in leaf and inflorescence tissues of the mock-inoculated samples of both genotypes. Panel D: DAB staining in inoculated Cabernet Cortis, reveals H_2O_2 accumulation on leaves in correspondence of the necrotic dots. Staining was not detectable on the inflorescence or on the Cabernet Sauvignon leaf and flower bottom.
Magnification bars correspond respectively to: 5mm, 3mm, 0,25mm and 5mm for Panel A; 1mm, 1mm, 0,5mm and 5mm for Panel B; 5mm, 3mm, 0,25mm and 3mm for Panel C; 0,5mm, 0,25mm, 0,5mm and 0,5mm for Panel D.

3.3.1.3. Ultrastructural analysis of the *P. viticola*–grapevine interaction

We used transmission electron microscopy (TEM) to further characterize the interaction between *P. viticola* and the two grapevine organs at ultrastructural level. In CC leaves we identified three areas with distinct responses to *P. viticola*: (i) close to pathogen sporulation, well-structured *P. viticola* hyphae and haustoria were present and plant cell organelles appeared slightly swelled and located close to the haustoria (Fig. 3.3C); (ii) at the border between green and brown spots, hyphae and haustoria were surrounded by massive deposit of paramural callose (arrow in Fig. 3.3D) that thickened the cell wall; (iii) the necrosis zones on the leaves, in which HR was activated and the tissue was degenerate, cells were showed a compact and electron-dense content and twisted cell walls, with cell structure and organelles (not shown). The areas characterized by the paramural callose raised interest, since callose synthesis is considered a grapevine induced defence response to DM (Kortekamp et al., 1997), and its deposition is related to resistant genotypes (Liu et al 2014, Yu et al 2012). Moreover, the role of callose in grapevine defence mechanisms was validated by the increase of the number of sporangia produced in leaf tissues infected with *P. viticola* treated with 2-deoxy-D-glucose (DDG), an inhibitor of callose synthesis. The increase of sporangia was observed also in *P. viticola* resistant variety Solaris, even thought Solaris treated tissues showed higher resistance compared to the basal resistance of susceptible Chasselas variety, indicating the involvement of further resistance factors, besides callose synthesis (Hamiduzzaman et al., 2005). Furthermore, callose deposition was observed on stomata as response to *P. viticola* infection and their percentage is used at 48 hpi as a histological marker to evaluate the degree of resistance to DM of grapevine varieties (Gindro et al., 2006). CC inflorescence tissues presented two types of pathogen-response zones: areas in which *P. viticola* haustoria were deformed or collapsed (Fig. 3.3E), and electron-dense necrosis areas characterized by mashed cells and twisted cell walls.

P. viticola was typically structured in both CS tissues, as showed in Fig. 3.3F-G for the leaf and 3.3H for the inflorescence. In the intercellular space of spongy parenchyma the pathogen presented hyphae vacuolated (Fig 3.3F and 3.3H) and several and well-structured haustoria (Fig. 3.3G and 3.3H). Grapevine tissues did not show evidence of necrosis or other alterations due to *P. viticola*. The spread of several and well-structured *P. viticola* hyphae and haustoria confirmed the compatible host-interaction in both susceptible CS organs and the establishment of biotrophy and the host-specificity, respectively (Unger et al. 2007, Díez-Navajas et al., 2008).

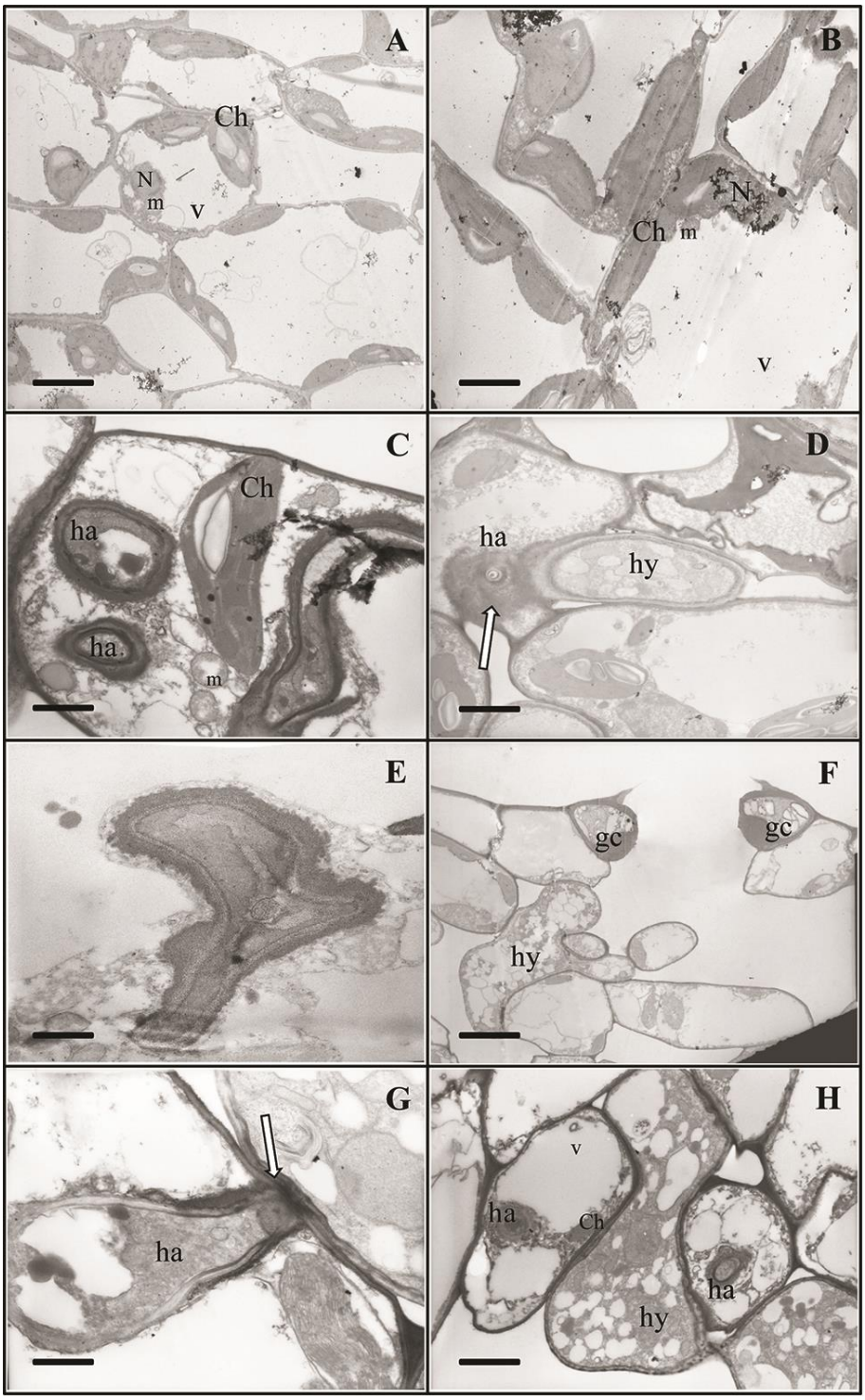


Fig. 3.3. Transmission electron micrographs of Cabernet Cortis and Cabernet Sauvignon leaf and inflorescence. A, B: control leaf and inflorescence tissues do not present ultrastructural alterations. C: leaf tissue of Cabernet Cortis in correspondence of *P. viticola* sporulation. Well-structured haustoria are recognizable inside a spongy parenchyma cell. D: in Cabernet Cortis leaf tissue at the border between green and brown spots haustoria are surrounded by massif deposit of paramural callose (arrow) that thickened the cell wall, hindering pathogen penetration. E: deformed and electron-dense *P. viticola* haustoria are found in Cabernet Cortis inflorescence tissues. F: *P. viticola* hyphae in the substomatal zone in Cabernet Sauvignon tissues. G: in Cabernet Sauvignon infected leaves haustoria show the typical pyriform shape. No callose deposition is visible at the haustorium neck (arrow). H, *P. viticola* hyphae in Cabernet Sauvignon inflorescence: as expected they are localized in the intercellular space of parenchyma and appear vacuolated. Magnification bars correspond to A, 400nm, B, 200nm, C, 100nm, D, 200nm, E, 50nm, F, 750nm, G, 100nm and H, 250nm.
(N = nucleus; Ch = chloroplast; v = vacuole; m = mitochondria; ha = haustoria; hy = hyphae)

3.3.2. Sequencing, mapping and analysis of the reads of Cabernet Cortis and Cabernet Sauvignon organs

Three biological replications of the CC and CS leaves and inflorescences were analysed at 48 hpi. The samples were collected from fruiting cuttings upon mock- and *P. viticola*-inoculation. On average, 20,372,879 reads with a length of 75 nt were obtained for each sample, of which 97.8 % was retained after quality filtering and adaptor removal (parsed reads; Table S3.3), and more than 81.34% of parsed reads mapped to the reference transcriptome. An average of 75% of reads mapped to unique locations, 10% aligned to more than one location and 15% did not aligned (Table S3.3). Reads mapped to unique locations were used to assess expression levels of grapevine and *P. viticola* genes (Table S3.4).

3.3.3. Identification and clustering of differentially expressed genes based on expression profiles

A total of 16,322 DEGs were found using DESeq (*Padj* ≤ 0.05; log2 fold-change ≥ 1.0) and were organized in 14 clusters based on their expression profile (Table S3.5, Fig. 3.4).

Cluster 1 comprised 665 and 434 genes constitutively differentially expressed in CCLC and CCIC compared to CSLC and CSIC, respectively. Cluster 2 included 5,960 genes constitutively differentially expressed in CCLC compared to CCIC and 6,202 genes of the CSLC compared to CSIC.

The clusters 3-8 consisted of genes up-regulated upon *P. viticola* inoculation, whereas the clusters 9-14 of genes which were down-regulated (Fig. 3.4). Groups 3-4 comprised genes differentially expressed in CC organs and not in the CS; particularly, 25 and 1157 DEGs were up-regulated in CCLI and CCII. In CS leaf and inflorescence – cluster 5 and 6 – respectively 201 and 18 genes were up-regulated. The cluster 7 included 213 up-regulated and common genes to all susceptible organs. The cluster 8 consisted of 188 up-regulated genes classified as generic response to pathogen attack. The clusters 9-10 comprised genes differentially expressed in CC organs and not in the CS; in detail, 5 and 1213 DEGs were down-regulated in CCLI and CCII. In CS leaf and inflorescence – cluster 11 and 12 – respectively 11 and 15 genes were down-regulated. The cluster 13 comprised 15 down-regulated and common genes to all susceptible organs. No gene was down-regulated in the generic response to pathogen attack (cluster 14) (Table S3.5, Fig. 3.4).

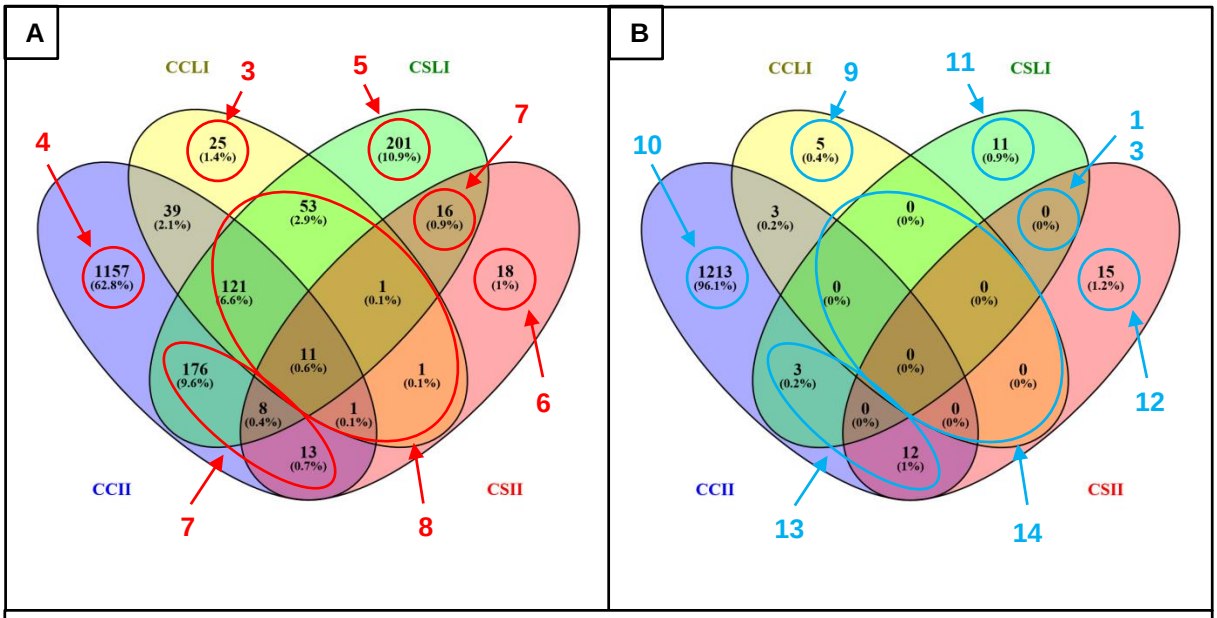


Fig. 3.4. Venn diagrams summarising the distribution of the 3061 differentially expressed genes (DEGs), upon *P. viticola* inoculation.
The 1802 up-regulated (Panel A) genes, were clustered in: Cluster 3, expressed only in CCLI; Cluster 4, expressed only in CCII; Cluster 5 expressed only in CSLI; Cluster 6, expressed only in CSII; Cluster 7, common to CCII, CSLI and CSII; Cluster 8, common to CCLI, CCII, CSLI and CSII.
The 1259 down-regulated (Panel B) genes, were clustered in: Cluster 9, expressed only in CCLI; Cluster 10, expressed only in CCII; Cluster 11 expressed only in CSLI; Cluster 12, expressed only in CSII; Cluster 13, common to CCII, CSLI and CSII; Cluster 14, common to CCLI, CCII, CSLI and CSII.

3.3.3.1. Constitutive transcriptional differences potentially related to basal resistance in Cabernet Cortis

Functions constitutively differentially regulated in CC leaf could potentially contribute to the partial resistance displayed by this organ. To date, differences in constitutive expression of DM resistance-related genes were analysed in the resistant *V. riparia* cv. 'Gloire de Montpellier' compared to the susceptible *V. vinifera* cv. 'Riesling' (Kortekamp, 2006). Therefore, to identify constitutive differences between the organs of CC and CS, expression levels of mock-inoculated samples were analysed using the CS organs as a reference since both are susceptible to *P. viticola* and genetically related to CC (Table S3.6).

262 genes with a constitutive higher expression in CCLC as compared with CSLC were identified. The following GO terms significantly over-represented ($P \leq 0.05$) were identified: signalling, response to stimuli (response to stress) and cellular process (Fig. 3.5A). Among genes related to signalling was identified a higher expression of a 12-

oxophytodienoate reductase 2 (*VIT_18s0041g02020*), a gene involved in JA biosynthesis. Probably, an higher content of this hormone was related to the mid-resistance of the CC leaf. Indeed, a new role for JA against fungal biotrophs (generally associated to the defence against necrotrophic pathogens and insects) was recently proposed and observed in the leaves of the resistant cv. 'Regent' by Guerreiro et al. (2016). The induction of JA was involved in the accumulation of secondary metabolites and PR proteins, in fact a constitutive higher expression of a pathogenesis-related protein 4-2 (PR-4-2) (*VIT_14s0081g00050*) and a chitinase (PR-3) (*VIT_04s0008g00120*), were found. The PR-3 and PR-4 proteins were related to the antifungal activity strengthening since they were able to hydrolyse the chitin, one of the components of cell wall of different fungi. On the other side, among genes belonging to secondary metabolism, particularly to phenylpropanoid biosynthesis, a ferulate 5-hydroxylase (*VIT_07s0031g01380*) was found suggesting an increased production of monolignols used for lignin production. This gene plays a critical role for the structure and physical properties of the lignin, as highlighted by Stewart et al. (2009) on a hybrid poplar. Moreover, 403 genes were identified with a constitutively lower expression in CCLC as compared with CSLC. Only the genes related to the biological regulation and response to stimuli (response to biotic stimuli and response to stress) were significantly over-represented ($P \leq 0.05$) in GO terms (Fig. 3.5B). Among genes involved in response to biotic stimuli were identified two osmotins (*VIT_02s0025g04250* and *VIT_02s0025g04260*) and three pathogen-related proteins (*VIT_18s0001g15660, VIT_03s0088g00710* and *VIT_05s0077g01580*). Interestingly, their expression increased much more in CCLI than in CSLI, upon *P. viticola* inoculation.

197 genes were identified with a constitutively higher expression in CCIC as compared with CSIC. No biological process functional and none of these genes appeared directly involved in genotype susceptibility. Furthermore, 237 genes were identified with a constitutively lower expression in CCIC as compared with CSIC. The only GO term significantly over-represented ($P \leq 0.05$) was the response to stimuli (stress) (Fig. 3.5C). Among these genes, four disease resistance proteins (*VIT_13s0067g00790, VIT_15s0046g02750, VIT_00s0226g00050* and *VIT_00s0238g00040*) were found, and their expression did not significantly vary upon pathogen inoculation.

To identify constitutive differences between the organs within the same genotype – for both CC and CS –, expression levels of the mock-inoculated samples were analysed using the respective inflorescence as reference (Table S3.6). Comparing the CCLC with CCIC, 3299 and 2018 genes were higher and lower expressed, respectively. Genes involved in

photosynthesis, generation of precursor metabolites and energy, metabolic process (protein metabolic process), gene expression (transcription) and cellular biosynthetic process were constitutively higher expressed in CCLC as compared to CCIC (Fig. S3.2A); while genes involved in localization (establishment of localization and transport), response to endogenous stimuli, carbohydrate metabolic process and secondary metabolic process were constitutively lower expressed in CCLC as compared to CCIC (Fig. S3.2B). Comparing the CSLC with CSIC, 2610 and 2081 genes were higher and lower expressed, respectively. Genes involved in cell communication, homeostatic process (cellular homeostatic process), regulation of biological quality, metabolic process (cellular protein metabolic process), photosynthesis and generation of precursor metabolites and energy were constitutively higher expressed in CSLC as compared to CSIC (Fig. S3.2C); while genes involved in localization (establishment of localization and transport), response to endogenous stimuli, biosynthetic process, transcription, carbohydrate metabolic process, reproductive developmental process and ripening were constitutively lower expressed in CSLC as compared to CSIC (Fig. S3.2D).

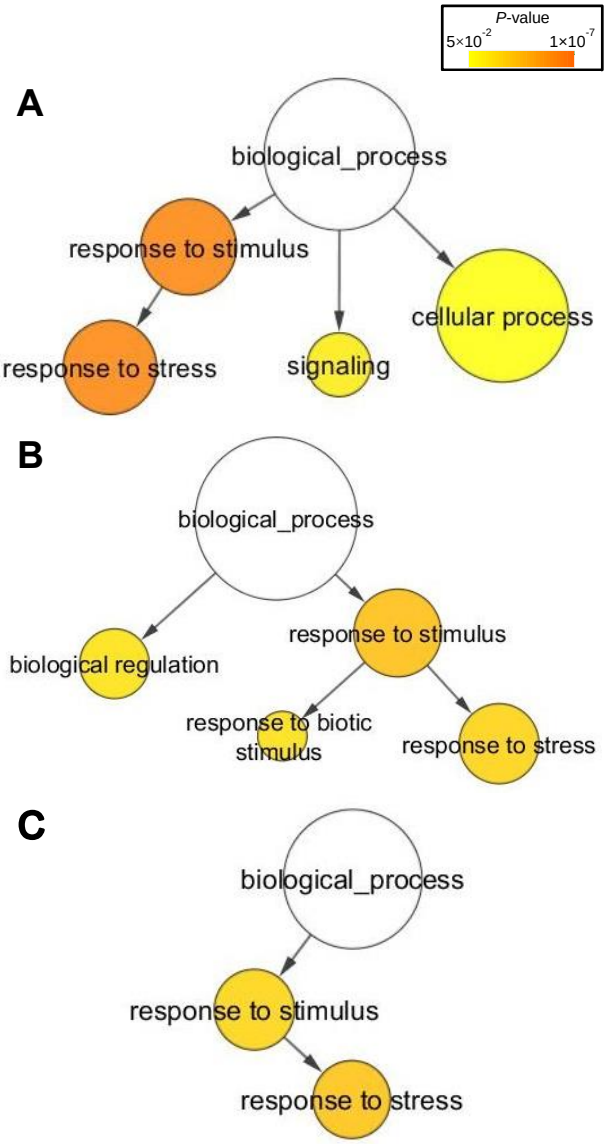


Fig. 3.5. Biological networks of significantly enriched Gene Ontology (GO) terms. GO biological process terms of the constitutively expressed genes in CC organs.
Panel A, constitutively higher expressed genes in CCLC compared to CSLC; Panel B, constitutively lower expressed genes in CCLC compared to CSLC; Panel C, constitutively lower expressed genes in CCIC compared to CSIC. No biological process functional was significantly enriched using genes higher expressed in CCIC compared to CSIC.
Enriched GO terms ($p < 0.05$) were identified using the BiNGO tool (Maere et al., 2005) and visualised with Cytoscape software (Shannon et al., 2003). The colour scale legend indicates the level of significance for enriched GO terms. White nodes indicate not significantly overrepresented categories.

3.3.3.2. Differentially expressed genes in *P. viticola*-inoculated leaves and inflorescences of Cabernet Cortis and Cabernet Sauvignon

Upon *P. viticola* inoculation, in CCLI (cluster 3 in Fig. 3.4) were up-regulated genes related to secondary metabolism (Fig. 3.6A). Interestingly, genes belonging to phytoalexin biosynthesis such as four stilbene synthase (*VIT_16s0100g01030*, *VIT_16s0100g01140*, *VIT_16s0100g00920*, *VIT_16s0100g00990*), and a pathogenesis related protein 1 precursor (*VIT_03s0088g00890*) were identified (Table S3.6). The up-regulation of stilbene synthases suggests the production increase of resveratrol and its oxidation products during *P. viticola* inoculation, as seen in several studies, particularly pterostilbene and viniferins (Bavaresco et al., 1997, Pezet et al. 2004, Gindro et al., 2006, Alonso-Villaverde et al., 2011, Mattivi et al., 2011) Indeed, δ-viniferin produced *in vitro* by the oxidative dimerization of resveratrol by plant peroxidases or fungal laccases, was identified in wines and in grapevine cell cultures (Langcake and Pryce 1977). δ-viniferin constitutes one of the most important phytoalexins derived from resveratrol, with a concentration higher than its isomer ε-viniferin (Pezet et al. 2003). Biochemical criteria, as δ- and ε-viniferin levels at the site of infection, were recorded at 48 hpi in seedlings to rapidly evaluate the level of DM resistance, thereby leading to a reduction in the breeding program duration by several years (Gindro et al. 2006). Likewise, a wall-associated receptor kinase-like 10 (*VIT_03s0132g00340*) and a glutathione S-transferase G 25 GSTU7 (*VIT_08s0040g00920*) were up-regulated. An increased expression of the former is associated with an amplified cGMP production, a second messenger carrying out a key role in signal transduction linking environmental stimuli to physiological responses, including biotic stress responses. Indeed in Arabidopsis, AtWAKL10 is consistently co-expressed with well characterized pathogen defence related genes and is induced early and sharply in response to a range of pathogens and their elicitors (Meier et al. 2010). On the other hand, the increased expression of glutathione S-transferase (GST) genes is related in the oxidative stress response and follows infection by several pathogens, as seen by Ahn et al (2016) on leaves of *V. flexuosa* inoculated with *Botrytis cinerea, E. ampelina,* and *Rhizobium vitis*. As a whole, the rapid activation of genes implicated in stilbene biosynthesis, genes encoding components of signal transduction cascades and defence-related genes is associated to the response of resistant genotypes after inoculation (Polesani et al, 2010; Wu et al., 2010; Malacarne et al., 2011; Figueiredo et al., 2012). All these genes are not modulated in susceptible genotypes and can be therefore associated to the early perception of the invading pathogen and to the activation

resistance mechanisms (Polesani et al., 2010; Malacarne et al., 2011; Figueiredo et al., 2012).

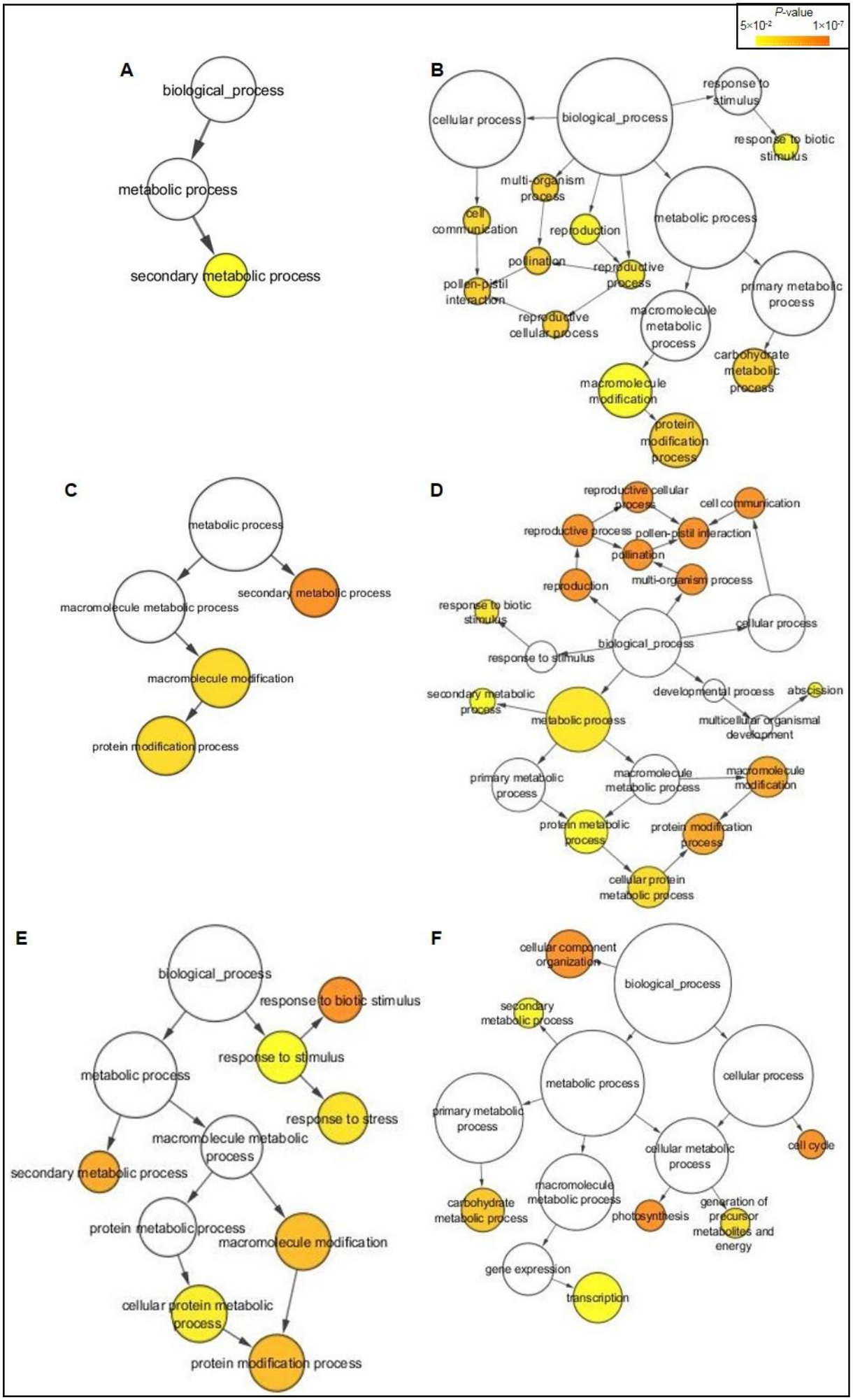
Genes implicated in the response to biotic stimuli, carbohydrate metabolic process, macromolecule modification (protein modification process), multi-organism process, cell communication and reproductive process (reproductive cellular process, pollination, pollen-pistil interaction), were up-regulated in CCII compared to CCIC (cluster 4 in Fig. 3.4; Fig. 3.6B and Table S3.6). Genes involved in the main hormone signalling pathways were up-regulated. In detail, two genes related to salicylic acid (SA) pathway – a phytoalexin-deficient 4 protein (PAD4) (*VIT_07s0031g02390*), and a senescence-associated gene 101 (SAG101) (*VIT_05s0077g01750*) were up-regulated, suggesting a SA pathway activation. However, a lack of the expression of the enhanced disease susceptibility 1 (EDS1) was found suggesting an uncompleted SA-mediated defence response. Indeed in Arabidopsis, EDS1 interacts with PAD4 and SAG101 in the cytoplasm and in the nucleus in distinct complexes and a nuclear pool of EDS1 is needed for resistance to biotrophic and hemibiotrophic pathogens (Feys et al. 2005, García et al. 2010, Rietz et al. 2011). Regarding jasmonate metabolism, two genes were identified: an allene oxide synthase (AOS) (*VIT_18s0001g11630*), that catalyzes the first step in the biosynthesis of JA and a MeJA esterase (*VIT_00s0253g00150*) involved in the biosynthesis of JA from MeJA. Additionally, a gene related to the cross-talk between ethylene and jasmonate signalling pathways – an ethylene response factor ERF1 (*VIT_05s0049g00510*) – was up-regulated. The activation of jasmonate signalling pathway in the first hours after *P. viticola* inoculation suggest a defence attempt in the inflorescence tissue against fungal biotrophs, highlighting its new role proposed for this hormone by Guerreiro et al (2016). Furthermore, signalling compounds, such as jasmonate, salicylate or ethylene can induce the expression of specific genes, known as pathogenesis-related (PR) genes (van Loon et al., 2006), involved in biotic stress response. In fact, the expression of some PR genes was up-regulated upon *P. viticola* inoculation, in particular a chitinase class IV (*VIT_05s0094g00340*), a beta-1,3-glucanase *(VIT_08s0007g06040*) and an endo-1,4-beta-glucanase (*VIT_18s0089g00210*), highlighting the attempt of this organ to respond to pathogen attack.

In CSLI compared to CSLC (cluster 5 in Fig. 3.4), the macromolecule modification (protein modification) and secondary metabolic process were over-represented (Fig. 3.6C). Upon *P. viticola* inoculation, was up-regulated a calmodulin binding protein (*VIT_17s0000g03380*), gene involved in cellular signalling cascades and in the defence response through the regulation of numerous target proteins (Ranty et al. 2006). However,

the up-regulation of this gene is in contrast with the specific induction of calmodulins and calmodulin-binding proteins in the resistant genotypes (Polesani et al. 2010, Figueiredo et al., 2012). No biological process functional was significantly enriched for cluster 6 (Fig. 3.4). Genes involved in response to biotic stimuli, metabolic process (secondary metabolic process, cellular protein metabolic process), macromolecule modification (protein modification), multi-organism process, cell communication and reproductive process (reproductive cellular process, pollination, pollen-pistil interaction) and abscission were significantly enriched for cluster 7 (Fig. 3.4, Fig. 3.6D). In the cluster 8, response to stimuli (biotic and stress), secondary metabolic process, macromolecule modification and cellular protein metabolic process (protein modification process) were significantly enriched (Fig. 3.4, Fig. 3.6E).

Among genes down-regulated, clusters 9, 11, 12, 13 and 14 (Fig. 3.4) did not present any significantly enriched categories. Conversely, cluster 10 presented enriched functional categories on photosynthesis, generation of precursor metabolites and energy, cell cycle, cellular component organization, secondary metabolic process, carbohydrate metabolic process and transcription (Fig. 3.6F). Altogether, the reduction of these biological processes could indicate a dramatic alteration of metabolism during the first 48 hours of *P. viticola* infection, with a source-to-sink transition. In particular, the alterations in starch and carbohydrate metabolism were observed in infected grapevine leaves at 7 dpi upon *P. viticola* inoculation (Gamm et al., 2011), while a strong accumulation of starch was observed at 24 and 48 hpi in leaves and inflorescences inoculated with *B. cinerea* and *B. pseudo cinerea* on fruiting cuttings of *V. vinifera*, suggesting that the metabolism of grapevine inflorescence and leaf is modified with distinct mechanisms in these two organs (Vatsa-Portugal et al. 2015). Interestingly, a down-regulation of the carbonic anhydrase, (*VIT_00s0252g00110*) and of the plastocyanin domain-containing protein (*VIT_12s0028g03450*) was found. Particularly import is the down-regulation of the carbonic anhydrase, an enzyme involved in the uncatalyzed interconversion between CO_2 and HCO_3 providing the carbon dioxide for fixation by RuBisCO (Badger & Price 1994). Its down-regulation could be required for the maintenance of a compatible interaction between *P. viticola* and grapevine, as seen on the susceptible leaves of *V. vinifera* cv. ‘Riesling’ by Polesani et al 2008. The enzyme has antioxidant activity, binds salicylic acid (Slaymaker et al., 2002), and the down-regulation of this gene was observed also in tomato plants following application of the fungal toxin fusicoccin (Frick and Schaller 2002), in Arabidopsis following treatment with MeJA (Schenk et al., 2000) and in potato infected with *P. infestans* (Restrepo

et al., 2005). The plastocyanin domain-containing protein (*VIT_12s0028g03450*) is a gene involved in electron transport from cit b6/f complex to the Photosystem I and its down-regulation could be associated with a reduction of the photosynthetic process. Likewise, a down-regulation of a precursor of the plastocyanin was observed in the leaf of susceptible genotype (Polesani et al. 2008).


P-value
5×10-2
1×10-7
A
biological_process
metabolic process
secondary metabolic process
B
cellular process
biological_process
response to stimulus
response to biotic stimulus
multi-organism process
cell communication
reproduction
metabolic process
pollination
reproductive process
pollen-pistil interaction
primary metabolic process
reproductive cellular process
macromolecule metabolic process
carbohydrate metabolic process
macromolecule modification
protein modification process
C
metabolic process
secondary metabolic process
macromolecule metabolic process
macromolecule modification
protein modification process
D
reproductive cellular process
cell communication
reproductive process
pollen-pistil interaction
pollination
reproduction
multi-organism process
response to biotic stimulus
cellular process
biological_process
response to stimulus
secondary metabolic process
developmental process
abscission
metabolic process
multicellular organismal development
primary metabolic process
macromolecule metabolic process
macromolecule modification
protein metabolic process
protein modification process
cellular protein metabolic process
E
biological_process
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response to stimulus
metabolic process
response to stress
macromolecule metabolic process
secondary metabolic process
protein metabolic process
macromolecule modification
cellular protein metabolic process
protein modification process
F
cellular component organization
biological_process
secondary metabolic process
metabolic process
cellular process
primary metabolic process
cellular metabolic process
cell cycle
macromolecule metabolic process
carbohydrate metabolic process
photosynthesis
generation of precursor metabolites and energy
gene expression
transcription

(See figure on previous page.)
Fig. 3.6. Biological networks of significantly enriched Gene Ontology (GO) terms. GO biological process terms of the differentially expressed genes (DEGs), upon *P. viticola* inoculation.
Panel A, up-regulated genes in CCLI compared to CCLC (Cluster 3); Panel B, up-regulated genes in CCII compared to CCIC (Cluster 4); Panel C, up-regulated genes in CSLI compared to CSLC (Cluster 5). Panel D, up-regulated genes in CCII, CSLI and CSII respectively compared to CCIC, CSLC and CSIC (Cluster 7); Panel E, up-regulated genes in CCLI, CCII, CSLI and CSII respectively compared to CCLC, CCIC, CSLC and CSIC (Cluster 8); Panel F, down-regulated genes in CCII compared to CCIC (Cluster 10). No biological process functional was significantly enriched for: CSII compared to CSIC (Cluster 6); CCLI compared to CCLC (Cluster 9); CSLI compared to CSLC (Cluster 11); CSII compared to CSIC (Cluster 12); CCII, CSLI and CSII respectively compared to CCIC, CSLC and CSIC (Cluster 13); CCLI, CCII, CSLI and CSII respectively compared to CCLC, CCIC, CSLC and CSIC (Cluster 14).
Enriched GO terms ($p < 0.05$) were identified using the BiNGO tool (Maere et al., 2005) and visualised with Cytoscape software (Shannon et al., 2003). The colour scale legend indicates the level of significance for enriched GO terms. White nodes indicate not significantly overrepresented categories.

3.3.4. Validation of the organ-specific response and RNA-Seq analysis

To validate the organ-specific response to *P. viticola* inoculation, the pathogen infection was confirmed with different evaluations methods, from macroscopic to ultrastructural, on an independent experiment.

In vivo P. viticola symptom assessment on CC organs confirmed the MR and MS respectively of the leaf and inflorescence (Fig. S3.3). PN showed a MS leaf with an OIV 452 score of 3.7 and an S inflorescence with an OIV 453 score of 2.3 (Fig. S3.3). Mus displayed both organs R to pathogen attack with an OIV 452 and OIV 453 score respectively of 7.7 e 7.3 (Fig. S3.3). Mock-inoculated organs had mean values of 9 for each organs and genotype analyzed.

The three genotypes presented a significant ($P \leq 0.05$) spread disease upon *P. viticola* inoculation on both organs (inoculated vs. mock) (Table S3.7A). Comparing the two organs in the same genotype only the CC showed significant phenotypic differences between leaf and inflorescence (Table S3.7A) owing to the divergent dual epidemics. Furthermore, the same organ between genotypes was compared using the susceptible PN as reference. Significant phenotypic differences between the CC and PN leaf as well as between Mus and PN organs were found (Table S3.7B), while the CC inflorescence did not show significant differences with PN (Table S3.7B). Differences or similarity were due to the diverse organ-specific response against the *P. viticola* inoculation.

Secondly, H_2O_2 production was analyzed through DAB staining and the reddish-brown areas were clearly detectable on CC leaf and on both Mus organs (red arrows in Fig. S3.4B).

No staining reaction was detected on CC inflorescence, PN organs as well as on mock-inoculated samples (Fig. S3.4A-B).

Finally, the TEM was used to investigate plant-pathogen interaction at ultrastructural level. In PN organs, the pathogen structures are well widespread in the intercellular space (hyphae) and in host cells (haustoria), and no plant reaction was detected (Fig. S3.5A-B). Ultrastructural observations of CC organs confirmed the presence of the different areas of response to *P. viticola* attack, as described above (Fig. 3.3C-E). Particularly, the leaf areas which showed well structured *P. viticola* hyphae and haustoria (Fig. 3.3C, Fig. S3.5D), and the leaf areas where the haustoria were totally surrounded by big collars of callose (Fig. 3.3D, Fig. S3.5C). In the inflorescence, *P. viticola* haustoria were deformed or collapsed (Fig. 3.3E). Mus had the same behaviour of the CC for both organs. In the second identified leaf areas, for instance, the haustoria appeared surrounded by callose collars and deformed (Fig. S3.5E). Interestingly, in the areas close to the necrosis zone the Mus showed phenolic compounds accumulation in the vacuoles. Such cellular alterations were present in both leaf and inflorescence tissues as black electron-dense structures (Fig. S3.5F). Moreover, the area appeared disorganized with collapsed cells, empty or with a warped vacuole (Fig. S3.5F). These cytological changes, including phenol deposition, cytoplasmic disorganization, and localized plant and pathogen cell death occurred specifically in cells invaded by the pathogen structures in the resistant grapevine hybrid Solaris as well as in the susceptible grapevine *V. vinifera* cv. ‘Marselan’ treated with Sulfated laminarin (PS3) (Trouvelot et al. 2008).

On the other hand, flavonols – constitutive phenolic compounds – are naturally synthesized and accumulated in grapevine leaves grown in vineyards with a primary role of protection against UV (Kolb et al. 2001; Kolb and Pfündel, 2005). In order to analyze the effect of flavonols and hydroxy-cinnamic acids on *P. viticola* infection, variable amounts of flavonols by different light conditions in phenologically identical leaves were induced; differences in content of leaf hydroxy-cinnamic acids were induced at the same time. Whatever the light condition, there were no significant changes in flavonol or in hydroxycinnamic acid contents for control and inoculated leaves during the development of *P. viticola* until 6 dpi. The implication of these leaf constitutive compounds in the defence of *V. vinifera* against DM was investigated *in vivo* thanks to the violet-blue autofluorescence of stilbenes used as an indicator of infection by *P. viticola*. It was revealed that the increase in stilbene violet-blue autofluorescence started earlier for leaves with low flavonol content than for leaves with higher content, suggesting that constitutive flavonols are able to lessen the

pathogen development; on the contrary, constitutive hydroxycinnamic acid does not seem to affect *P. viticola* infection (Latouche et al. 2013).

Besides leaves, experiments have been carried out also at inflorescence/bunch level. Clusters of two grapevine genotypes, Chasselas and Merlot, and two inter-specific hybrids, Solaris and 2091, were inoculated with *P. viticola* at different developmental stages. Microscopic examinations of pedicels, rachis and calyptras showed important differences in stomatal structures within seasonal development. At the stage of visible inflorescences, successful infections were observed on all tested cultivars and functional stomata were present, while no infections were observed after this stage. Significant stilbene accumulation was quantified in resistant hybrids which produced pterostilbene and δ-viniferin in large amounts, whereas in the susceptible varieties, only piceid and resveratrol were induced (Gindro et al. 2012). Lately, the phenolic composition of grape berries of other hybrids (CC, Johanniter, Solaris, Phoenix, and Regent) was analyzed using two complementary Liquid Chromatography - Tandem Quadrupole Mass Spectrometry (LC-MS/MS) methods. Resistant or tolerant hybrids contain higher level of phenolics in respect to *V. vinifera* cultivars, especially in the class of stilbenes (Ehrhardt et al. 2014).

3.4. CONCLUSIONS

In this study, we characterized genes belonging to enriched biological functions that may be associated with either mid-resistance or mid-susceptibility/susceptibility to DM (e.g. biotic and oxidative stress responses, hormone signalling, defence-related genes, signal transduction, carbohydrate metabolism and photosynthetic process), and whose expression is organ-specific in CC and CS tissues.

The next step will be the validation of the RNA-Seq results testing the selected genes by RT-qPCR using the 2 grapevine housekeeping genes to normalise the gene expression and the *P. viticola* actin to carry out a relative *P. viticola* biomass accumulation. The final step will be to compare their expression profiles at inter-genotype level using the independent experiment on Mus and PN.

Since, the *P. viticola* draft genome sequence have been recently released (Dussert et al. 2016), one of the future objective of this research work will be to analyse *P. viticola* DEGs in the CC and CS organs as well. DM gene prediction and annotation can take advantage of the sequenced genomes within the *Phytophtora* genus, an oomycete phylogenetically very close to *Plasmopara* (Tyler et al. 2006, Haas et al. 2009, Feau et al. 2016). This milestone opens the way towards the full understanding of the pathogen itself and in the grapevine-*P. viticola* interaction.

3.5. ACKNOWLEDGEMENTS

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3.6 APPENDIX 2

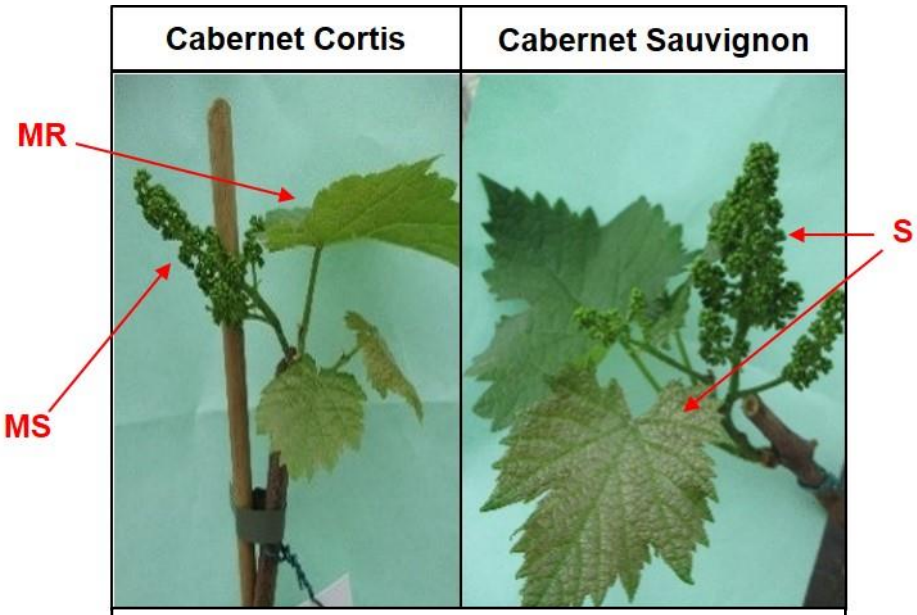

Fig. S3.1. Fruiting cutting production: genotypes of interest and downy mildew response level on leaf and inflorescence.

Fig. S3.2. Biological networks of significantly enriched Gene Ontology (GO) terms. GO biological process terms of the constitutively expressed genes in CC and CS organs.
Panel A, constitutively higher expressed genes in CCLC compared to CCIC; Panel B, constitutively lower expressed genes in CCLC compared to CCIC; Panel C, constitutively higher expressed genes in CSLC compared to CSIC; Panel D, constitutively lower expressed genes in CSLC compared to CSIC.
Enriched GO terms ($p < 0.05$) were identified using the BiNGO tool (Maere et al., 2005) and visualised with Cytoscape software (Shannon et al., 2003). The colour scale legend indicates the level of significance for enriched GO terms. White nodes indicate not significantly overrepresented categories.

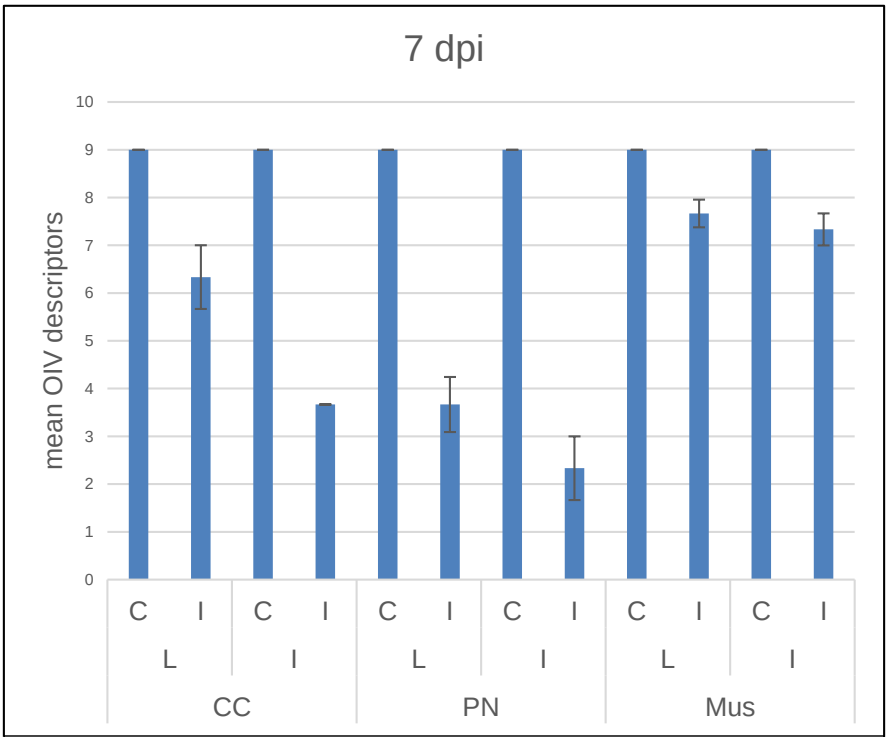

Fig. S3.3. Means of OIV452/453 descriptors scores of Cabernet Cortis (CC), Pinot Noir (PN) and Muscaris (Mus) respectively on leaf (L) and inflorescence (I) at 7 days post infection (dpi). The histogram reports the mean scores of mock- (C) and *P. viticola*-inoculated (I) samples.

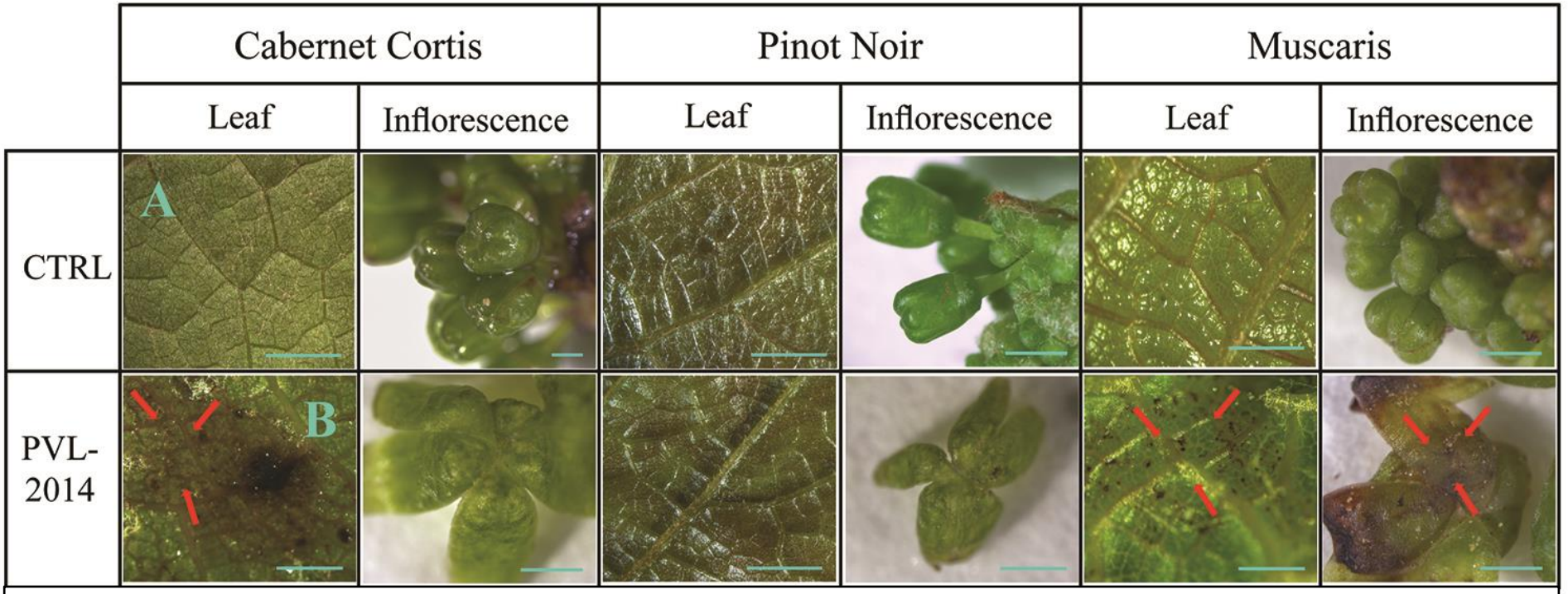

Fig. S3.4. DAB staining of control (panel A) and *P. viticola*-inoculated (panel B) grapevine leaves and inflorescences.
Panel A: in Cabernet Cortis, Pinot Noir and Muscaris mock-inoculated samples no DAB staining is observed. Panel B: DAB staining in inoculated Cabernet Cortis leaf and Muscaris organs, reveals H_2O_2 accumulation in correspondence of the necrotic dots (red arrows). Staining was not detectable on the Cabernet Cortis inflorescence and on the Cabernet Sauvignon tissues. Magnification bars correspond to 5mm, 1mm, 5mm, 0,3mm, 5mm and 0,3mm for Panel A; 0,5mm, 0,25mm, 5mm, 0,5mm, 0,5mm and 0,25mm for Panel B.

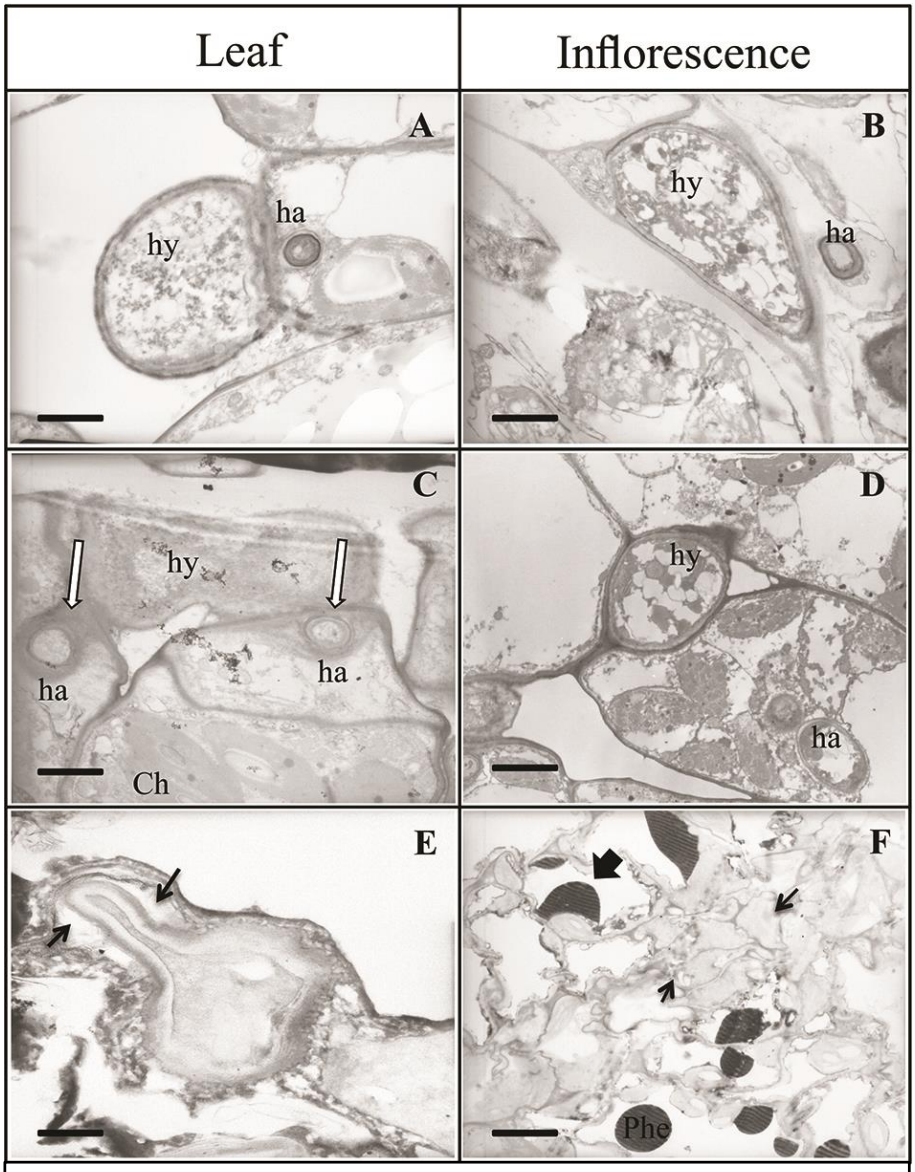

Fig. S3.5. Transmission electron micrographs of Pinot Noir, Cabernet Cortis and Muscaris leaf and inflorescence.
A and B, Pinot Noir tissues: *P. viticola* hyphae and haustoria are well structured and widespread in the intercellular spaces (hyphae) and inside host cells (haustoria). C and D, Cabernet Cortis leaves: C: the haustoria (ha) are surrounded by big paramural callose depositions (arrows); D: *P. viticola* hyphae and haustoria are normally developed and structural reactions by plant host are not visible. E and F, Muscaris tissues. E: Callose is visible close to a deformed haustorium (arrow). In leaf tissues, close to the necrosis zone, vacuolar phenolic accumulation are detected (F). Magnification bars correspond to A, B and C, 150nm, D, 250nm, E, 70nm and F, 400nm.
(Ch = chloroplast; ha = haustoria; hy = hyphae; Phe = phenolic compounds).

3.7. Supplementary material available upon request

Table S3.1. Non-redundant dataset of 99 *P. viticola* cDNA sequences, followed by their accession number, coding DNA sequence (cds) and authors. Sequences obtained from Reference sequence (RefSeq) database at NCBI (https://www.ncbi.nlm.nih.gov/nuccore/?term=Plasmopara%20viticola).

Table S3.2. Target genes and primer sequences for quantitative real-time RT-PCR (RT-qPCR) expression analysis of *P. viticola* and grapevine genes.

Table S3.3. Summary of RNA-seq sequencing data and mapping metrics.

Table S3.4. Expression levels of grapevine and *P. viticola* genes.

Table S3.5. Summary of the clustering of differentially expressed genes (DEGs).

Table S3.6. Clustering and functional annotation results of differential expressed genes (DEGs).

Table S3.7. Comparisons between the evaluated parameters on leaf and inflorescence of Cabernet Cortis, Muscaris and Pinot Noir. Panel A: different organs within the same genotype. Panel B: the same organ between different genotypes (— = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, / = not calculated).

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CONCLUSIONS AND FUTURE PROSPECTIVES

In agricultural settings, dual epidemics are pathogen infections that develop on two or more plant organs in the course of a cropping season. Dual epidemics are particularly important when one of the organs affected by the disease constitutes the harvestable part of the plant, and are known in several pathosystems with important economic implications (e.g. apple scab, strawberry powdery mildew and rice blast). Grapevines are host of a variety of economically important pathogens, some of which differentially infect the vegetative and reproductive organs, decreasing yield and fruit quality (e.g. grey mould, powdery and downy mildew, and *Phylloxera*). Grapevine dual epidemics are often complex to manage, because the association between epidemiological components occurring on different organs has been scarcely investigated, and because to foresee the risk toward the harvestable grapes has been barely attempted.

Based on a phenotypic screening under field and controlled conditions, the *Vitis* hybrid CC was identified as a model to study divergent dual epidemics in DM, since displays mid-resistant leaves and mid-susceptible inflorescences. Indeed, the diverse DM epidemiological behaviour at organ level observed *in vivo* was confirmed both by organs detached from fruiting cuttings and by the phenotypic evaluations on overall plants grown in the greenhouse. Therefore, the changes induced by *P. viticola* were investigated by combining phenotypic, histological, ultrastructural and transcriptomic approaches, in order to provide comprehensive information about the different organ-response. The macroscopic, microscopic and ultrastructural response to DM showed that in the mid-resistant CC leaf the sporulation was related to the H_2O_2 production and to the callose deposition in specific areas, while in the mid-susceptible CC inflorescence they were not related each other.

Through the transcriptomic analysis, genes belonging to enriched biological functions that may be associated with either mid-resistance or mid-susceptibility/susceptibility to DM, and whose expression is organ-specific in CC tissues were identified. In particular, the mid-resistant CC leaf was characterised by an up-regulation of genes implicated in biotic and oxidative stress responses, signal transduction and defence-related genes. Genes implicated in the hormone signalling were up-regulated in mid-susceptible CC inflorescence, while genes related to carbohydrate metabolism and photosynthetic process were down-regulated. This study provides a first exploration of the functions associated with varying levels of partial resistance to DM in grapevine organs that can be exploited as sources of genetic resistance in grapevine breeding programs.

The next step will be the validation of the RNA-Seq results testing the selected genes by RT-qPCR and the analysis of their expression profiles at inter-genotype level using the independent experiment on the hydrid Mus and the variety PN. One of the future objective of this research work will be to analyse *P. viticola* differential expressed genes in the grapevine organs, to have a better understanding of the pathogen itself and in the grapevine-*P. viticola* interaction.

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