DISS. ETH NO.21125

FACTORS AFFECTING THE *TRICHODERMA HARZIANUM* -INDUCED RESISTANCE AGAINST DOWNY MILDEW OF GRAPEVINE AND INTERACTION BETWEEN *PLASMOPARA VITICOLA* GENOTYPES CO-INOCULATED IN THE HOST

A dissertation submitted to

ETH ZURICH

For the degree of

Doctor of Sciences

Presented by

BENEDETTA ROATTI

MSc, Università di Firenze

Born January 7th, 1983

Citizen of Bologna (IT)

Accepted on the recommendation of

Prof. Dr. Cesare Gessler, examiner

Prof. Dr. Bruce A. McDonald, co-examiner

Prof. Dr. Brigitte Mauch-Mani, co-examiner

Dr. Ilaria Pertot, co-examiner

2013

TABLE OF CONTENTS

SUMMARY 1

RIASSUNTO 3

CHAPTER 1 5
General introduction

CHAPTER2 21
Co-inoculated *P. viticola* genotypes compete for the infection of the host independently from the aggressiveness components

CHAPTER 3 38
Abiotic stresses affect *Trichoderma harzianum* T39-induced resistance to downy mildew in grapevine

CHAPTER 4 66
Characterization of resistance mechanisms activated by *Trichoderma harzianum* T39 and benzothiadiazole to downy mildew in different grapevine cultivars

CHAPTER 5 90
General conclusions

AKNOWLEDGEMENTS 98

PUBLICATIONS 99

CURRICULUM VITAE 100

SUMMARY

Grapevine (*Vitis vinifera* L.) is one of the major fruit crops worldwide and varieties used for table grape or wine production are susceptible to several pathogens. Downy mildew caused by the oomycete *Plasmopara viticola* is an important grapevine disease that threatens leaves and young berries and, to avoid yield losses, control of the disease is based on the application of chemical fungicides. Genetic analysis of pathogen's population structure in field indicated that germination of oospores, causing primary infections occurs for a long period, alongside secondary infection cycles, and few *P. viticola* genotypes are dominating during an epidemic. Alternative methods for controlling downy mildew have been studied, including the use of microbial biocontrol agents. Application of *Trichoderma harzianum* T39 (T39) has been shown to reduce downy mildew symptoms in grapevine by activating the plant mediated resistance mechanism. Induced resistance offers the prospect of broad-spectrum disease control using the plant's own defenses and represents a promising low-impact tool for controlling crop diseases. However, the potential of induced resistance has yet to be fully realized, mainly due to its only partial control of the disease and its inconsistency under field conditions. Induced resistance is a plant-mediated mechanism, and its expression under field conditions is likely to be influenced by a number of factors, including environment, genotype and crop nutrition. Concerns about the impact of abiotic stresses on agriculture have been raised in the last decade, especially in light of the predicted effects of climate changes. High temperatures and drought associated with heat waves may occur with increased frequency as a result of climate change, threatening crop production and influencing interactions with both pathogenic and beneficial microorganisms. Aim of this project was to study the downy mildew disease form the pathogen and from the host plant point of views, in order to maximize the control of the disease with low-impact natural methods. To evaluate the efficacy of the T39-induced resistance under non-optimal conditions and to study pathogen's infection dynamics of different *P. viticola* isolates we evaluated i) the T39-induced resistance in plants exposed to heat and drought stresses, ii) the T39-induced resistance in different grapevine cultivars and iii) the possible selection mechanisms of different *P. viticola* isolates. The work was structured in three distinct sections where, at first, we could assess that co-inoculated *P. viticola* isolates competed for the infection of the host, although being equally infective when singularly inoculated. Competition was not related to the origin of the isolate and we hypothesized that competitive selection was modulated by differences in the

secretion of effector molecules, which explained the establishment of dominant genotypes over an epidemic season. In other two sections we demonstrated that T39-induced resistance was found to be reduced in plants exposed to the combination of heat and drought stresses, moreover, variable levels of efficacy were observed in different grapevine cultivars. Modulation of the marker genes in the T39-induced resistance was partially attenuated in plants under heat and drought stress. The molecular mechanisms activated in response to the resistance inducer were different and complex among cultivars, indicating that specific receptors are probably involved in the regulation of the plant response. The work presented in this thesis provides a deeper understanding to current knowledge of the biology of this grapevine pathogen and of the mechanisms of the induced resistance. Different *P. viticola* genotypes reacted differently when co-existing on a unique substrate, indicating that plant-pathogen interactions seem to be more complex than mere colonization of the plant tissue. The environmental conditions and the plant genotype are key factors affecting the T39-induced resistance. Therefore, prevention of predominant genotypes during an epidemic season and consideration of the variable responsiveness of the plant to the resistance inducer applied are important strategies for the improvement of biocontrol methods against downy mildew of grapevine.

RIASSUNTO

La Vite (*Vitis vinifera* L.) è una delle più importanti piante da frutto a livello mondiale e le varietà utilizzate per la produzione di uva da tavola o vino sono suscettibili a diversi agenti patogeni. La peronospora della vite, causata dall'oomicete *Plasmopara viticola,* è una malattia della vite importante che minaccia le foglie e i giovani grappoli e il controllo della malattia, per evitare ingenti danni alla produttività, si basa sull'applicazione di fungicidi chimici. L'analisi genetica della struttura della popolazione del patogeno in campo ha indicato che la germinazione delle oospore, causa di infezioni primarie, si verifica per un lungo periodo, in concomitanza con i cicli di infezione secondaria, e pochi genotipi di *P. viticola* prevalgono durante un'epidemia. Metodi alternativi per il controllo della peronospora sono stati studiati, compreso l'uso di agenti di biocontrollo microbici. L'applicazione di *Trichoderma harzianum* T39 (T39) ha dimostrato di ridurre i sintomi di peronospora della vite, attivando il meccanismo di resistenza indotta delle piante. La resistenza indotta offre una prospettiva di controllo della malattia ad ampio spettro sfruttando le difese della pianta e rappresenta un promettente strumento a basso impatto ambientale per il controllo delle malattie delle colture. Tuttavia, il potenziale di resistenza indotta deve ancora essere pienamente realizzato, principalmente per il controllo solo parziale della malattia e la incongruenza in condizioni reali. La resistenza indotta è un meccanismo mediato dalla pianta, e la sua espressione in condizioni reali può essere influenzato da una serie di fattori, tra cui l'ambiente, il genotipo e lo stato di nutrizione delle colture. Le preoccupazioni per l'impatto degli stress abiotici sull'agricoltura sono state sollevate negli ultimi dieci anni, soprattutto alla luce degli effetti previsti dei cambiamenti climatici. Alte temperature e siccità, associate con le ondate di calore, possono verificarsi con maggiore frequenza a causa del cambiamento climatico, minacciando la produzione delle colture e influenzando le interazioni sia con i patogeni sia con microrganismi benefici. Scopo di questo progetto è stato quello di studiare la peronospora della vite dal punto di vista della pianta ospite e del patogeno al fine di massimizzare il controllo della malattia con metodi naturali a basso impatto. Per valutare l'efficacia della resistenza indotta da T39 in condizioni non ottimali e per studiare le dinamiche di infezione in diversi isolati di *P. viticola* abbiamo valutato i) la resistenza indotta da T39 nelle piante esposte a stress termico e siccità ii) la resistenza indotta da T39 in diverse varietà di vite e iii) i possibili meccanismi di selezione in diversi isolati di *P. viticola*. Il lavoro è stato strutturato in tre sezioni distinte in cui, in un primo momento, abbiamo potuto valutare che isolati di *P.*

viticola co-inoculati competono per l'infezione dell'ospite, pur essendo ugualmente infettivi quando singolarmente inoculati. La concorrenza non è correlata all'origine degli isolati e abbiamo ipotizzato che la selezione competitiva fosse modulata da differenze nella secrezione di molecole effettrici, che spiegano la stabilizzazione di genotipi dominanti in una stagione epidemica. Nelle altre due sezioni, abbiamo dimostrato che la resistenza indotta da T39 è stata trovata ridotta in piante esposte alla combinazione di stress termico e siccità, inoltre, livelli variabili di efficacia sono stati osservati nelle diverse varietà. La modulazione dei geni marcatori della resistenza indotta da T39 risulta parzialmente attenuata nelle piante stressate con il calore e la siccità. I meccanismi molecolari attivati in risposta alla resistenza indotta sono diversi e complessi fra le varietà di vite, indicando che recettori specifici sono probabilmente coinvolti nella regolazione della risposta della pianta. Il lavoro presentato in questa tesi fornisce una comprensione più profonda delle conoscenze sulla biologia di questo patogeno della vite e sui meccanismi di resistenza indotta. Diversi genotipi di *P. viticola* reagiscono diversamente quando co-esistono su un unico substrato, indicando che le interazioni pianta-patogeno sembrano essere più complesse di una semplice colonizzazione del tessuto vegetale. Le condizioni ambientali e il genotipo di pianta sono fattori chiave che influenzano la resistenza indotta da T39. Pertanto, la prevenzione di genotipi predominanti nel corso di una stagione epidemica e la considerazione delle variabili risposte della pianta all'induttore di resistenza applicato sono importanti strategie per il miglioramento dei metodi di lotta biologica contro la peronospora della vite.

Chapter 1

General introduction

Downy mildew of grapevine

The causal agent of downy mildew is *Plasmopara viticola* (Berck. and Curt.) Berl. and de Toni, a heterothallic (Wong et al., 2001) diploid oomycete and obligate biotrophic parasite (Agrios 1997). *P. viticola* is native to North America, where is endemic on wild *Vitis* species (Gessler et al., 2011). It was first observed in Europe in 1878 and introduced with the importation of American grape cuttings used to replant the French vineyards destroyed by phylloxera (Rumbou and Gessler 2004). Since then, the disease was rapidly identified in many different regions from Easter toward Western Europe, reaching also southern regions such as Greece and Turkey (Gessler et al., 2011). In the 20th century, the disease was clearly a huge problem for the European viticulture until the end of the Second World War, when the grape-producing industry was able to avoid large-scale losses thanks to the widespread use of highly effective chemical control measures (Gessler et al., 2011).

P. viticola uses both sexual and asexual reproduction style and the life cycle begins when, in spring, oospores germinate producing macrosporangia. The oospores are sexually-produced at the end of the summer/begin of autumn of the previous year and they overwinter in the fallen leaves or in any infected organs residues (Gessler et al., 2011). The germinating oospores release macrosporangia that can be spread directly onto leaves by rain splash and turbulence, and the zoospores contained inside are released causing the primary infections (Gessler et al., 2011). Two flagella permit to the zoospores to swim in the water film on the lower side of the leaf surface. In proximity of a stomata they encyst on it forming a germ tube which penetrate the leaf tissues through the stomatal aperture (Agrios 1997). All green parts of the plants which have active stomata can be colonized by the zoospores and symptoms are easily recognized as yellowish lesions on leaves, known as “oil-spots”. When conditions are favourable, sporangiophors (carrying microsporangia) are produced from the primary lesion and this phase correspond to the appearance of the characteristic white mould at the lower side of the leaf (Agrios 1997). The microsporangia contain asexually-produced zoospores which, in presence of leaf-wetness, infect other parts of the plant causing secondary infections (Gessler et al., 2011). Numerous secondary infections, alongside primary infections, may occur in one season depending on the rain events and on the presence of dew (Gobbin et al., 2006) leading to abrupt increase in disease severity with a disastrous impact on grape yield (Rumbou and Gessler 2004).

Genetic structure of populations during an epidemic

The basic assumption on epidemic development of downy mildew asserted that the time-frame for oospores to germinate and cause primary infections was less than one month and that the supply of oospores was depleted shortly after bloom, thus contributing to the downy mildew epidemic only as initial inoculum source (Gessler et al., 2011; Rumbou and Gessler 2004). The secondary cycles were thought to be the responsible for the growth of an epidemic and the dispersal of the propagules was considered to cover long distances (Rumbou and Gessler 2004). Based on these assumptions, control strategies design and modelling programs were formed, however, they failed to provide effective control strategies (Rumbou and Gessler 2004). The role of the oosporic infection in the epidemic was reconsidered when several assays indicated that the time-frame for oospores germination covered a long period (Gessler et al., 2011). Step forward in the analysis of the contribution of primary and secondary infections during an epidemic came with the development of genetic tools that allowed the genotypic characterization of the lesions. Thus, it became possible to determine whether an epidemic was caused by few primary infections (Gessler et al., 2011). Population genetic studies with microsatellite markers (SSR) have shown that, over the course of the season, slowly increasing numbers of clonal genotypes (secondary infections) were observed, but new genotypes continued to appear as well, proving that new oosporic infections play an important role in downy mildew epidemic (Gobbin et al., 2007; Gobbin et al., 2005; Gobbin et al., 2003; Gobbin et al., 2003; Gobbin et al., 2006; Rumbou and Gessler 2004; Rumbou and Gessler 2006). Moreover, the success of the secondary sporangia in causing infection was low and covering short distances (Matasci et al., 2010; Rumbou and Gessler 2004). Thus, in contrast with the existing belief that primary infections occur only at early disease stages and in limited scale, it was shown that oosporic infections play a main role at the initiation of the disease and occur throughout the epidemiological season, with most of the genotypes having a limited ability to spread asexually (Gobbin et al., 2007; Gobbin et al., 2005; Gobbin et al., 2006; Rumbou and Gessler 2004; Rumbou and Gessler 2006). Another interesting aspect found during the analysis of population structure in fields was that, while the majority of genotypes fail to reproduce further after primary infections, some of them have been found to

produce secondary lesions and to predominate in summer populations of the pathogen (Gobbin et al., 2006; Rumbou and Gessler 2004).

Predominant genotypes

The origin and the causes of the selection of the predominant genotypes have scarcely investigated, however, several plausible hypothesis have been made. Difference in aggressiveness among isolates has been hypothesized to be relevant in genotypes selection. In fact, *P. viticola* isolates with values of aggressiveness components (latent period, infection frequency, spore production) higher than others are predominating in co-inoculation experiments (Corio-Costet et al., 2011). Thus, differences in aggressiveness would explain the differences in frequencies of various genotypes in *P. viticola* populations observed during the season. Another hypothesis argues that differences in aggressiveness among isolates may be greater under non-optimal conditions (Pariaud et al., 2009), suggesting that differential responses in terms of aggressiveness may exist between genotypes originating from different climatic regions. Apart differences in aggressiveness, a third hypothesis to explain the selection of predominant genotypes came observing competitive interactions between isolates in other classes of Oomycetes. When *Phytophthora infestans* isolates were co-inoculated in a competitive situation, competitive interactions were observed and it has been hypothesized that the isolate inducing defense proteins in the host plant earlier than others would have the competitive advantage (Young et al., 2009).

Mechanisms of plant resistance and susceptibility to the disease

The grapevine industry relies predominantly on cultivars that belongs to the *Vitis vinifera* specie which are highly susceptible to the disease (Perazzolli et al., 2012). On the contrary, the wild species exhibit variable levels of resistance (Gessler et al., 2011). The constitutive resistance of wild *Vitis* spp. to *P. viticola* is linked to the presence of specific resistance genes (Moreira et al., 2011) and to the high levels of expression of antimicrobial compounds (Borie et al., 2004; Figueiredo et al., 2008). Microscopic observations and transcriptional studies revealed that first steps of the infection process are essentially the same in susceptible and resistant grapevines, indicating that downy mildew resistance is mainly a post-infection phenomenon

(Dìez-Navajas et al., 2008; Kortekamp and Zyprian 2003; Polesani et al., 2010; Unger et al., 2007). The mechanisms of post-infection resistance after *P. viticola* inoculation include callose deposition in stomata (Gindro et al., 2003), cell wall-associated defence process (Dìez-Navajas et al., 2008; Jürges et al., 2009), accumulation of antimicrobial compounds such as resveratrol and viniferins (Dai et al., 1995) hypersensitive response (HR) (Bellin et al., 2009; Dìez-Navajas et al., 2008; Kortekamp 2006; Kortekamp and Zyprian 2003), accumulation of reactive-oxygen species and increased peroxidase activity (Kortekamp and Zyprian 2003). Moreover, a rapid up-regulation of genes coding for pathogenesis-related proteins (PR) (Kortekamp 2006) and activation of defence-related signal transduction were observed in resistant species (Kortekamp 2006; Polesani et al., 2010). In *V. vinifera* spp. a compatible interaction between host and pathogen is probably achieved through a lack of recognition by the plant (Gessler et al., 2011; Polesani et al., 2010). The failure of susceptible plants to mount an effective defence response is likely due to the fact that their resistance gene alleles do not develop into any *P. viticola*-specific recognitions system, since *V. vinifera* did not evolve in the presence of *P. viticola* (Di Gaspero et al., 2007). Transcriptional changes associated with early stages of *P. viticola* infection indicate that a weak defence response is activated in susceptible cultivars, and transcripts from all major functional categories, including defence processes, are strongly down-regulated at the oil-spot stage (Polesani et al., 2010; Polesani et al., 2008).

Induced resistance in plants: general mechanisms

The development of substances able to stimulate the natural defences in plants against pathogen's attack is one of the most ambitious goals in modern agriculture. The molecules or microorganisms able to activate the resistance in plants are called elicitors (Boller and Felix 2009). The elicitors can activate two different types of resistance in plants: the systemic acquired resistance (SAR) or the induced systemic resistance (ISR) (Boller and Felix 2009; Pieterse et al., 2009). Whereas SAR is typically mediated by salicylic acid (SA) and is linked to the production of PR proteins upon pathogen attack (Pieterse et al., 2009), ISR is activated by specific strains of beneficial soil-borne microorganisms or other non-pathogenic microorganisms or their metabolites (Shoresh et al., 2010; Walters 2009) through root or leaf interaction (Pieterse et al., 2000). Usually it involves the activation of a priming state for an accelerated jasmonic acid (JA) and ethylene (ET) dependent gene expression, which becomes evident only after pathogen attack

(Conrath et al., 2006; Pieterse et al., 2009). In the ISR system, primed plant defences are activated only when they are really needed (Verhagen et al., 2004) and provides advantages in terms of energy costs for the plant (van Hulten et al., 2006), thus, priming probably evolved to allow the plant to conserve energy under pathogen-free conditions (Walters and Heil 2007).

Resistance inducing agents against downy mildew of grapevine

Several substances with the ability to increase grapevine resistance to downy mildew were identified, such as chitosan (Aziz et al., 2006), laminarin (Aziz et al., 2003), sulphated laminarin (Allègre et al., 2009; Trouvelot et al., 2008), oligogalacturonide (Allègre et al., 2009), β-aminobutyric acid (BABA) (Dubreuil-Maurizi et al., 2010; Hamiduzzaman et al., 2005; Reuveni 2001) and acibenzolar-S-methyl (BTH) (Perazzolli et al., 2008). In addition to chemical inducers, plant extracts, aqueous extract of *Penicillum chrisogenum* (Thuerig et al., 2006), extracts of *Solidago Canadensis*, *Aureobasidium pullulans* (Harm et al., 2011) and the microorganism *Trichoderma harzianum* strain T39 were able to increase grapevine resistance to downy mildew (Perazzolli et al., 2008). Mechanisms of induced resistance against downy mildew of grapevine have been shown to involve stomatal closure (Allègre et al., 2009), expression of defence genes (Hamiduzzaman et al., 2005; Harm et al., 2011; Perazzolli et al., 2011; Trouvelot et al., 2008), increased enzymatic activity (Godard et al., 2009), callose deposition (Hamiduzzaman et al., 2005) and accumulations of phytoalexins (Dercks and Creasy 1989; Ferri et al., 2009; Godard et al., 2009; Slaughter et al., 2008). Priming was described following treatment with BABA (Dubreuil-Maurizi et al., 2010; Hamiduzzaman et al., 2005), sulphate laminarin (Trouvelot et al., 2008) plant extracts (Godard et al., 2009) and fosetyl-Al (Dercks and Creasy 1989).

Resistance inducers used in this study: BTH and Trichoderma harzianum T39

The most commonly used chemical SAR inducer is the benzothiadiazole derivate benzo (1,2,3)thiaziadole-7-carbothioic acid S-methyl ester (BTH), also known as acibenzolar-S-methyl (Perazzolli et al., 2008). This chemical is registered and applied on several crops under the brand name Bion® (Syngenta) (Perazzolli et al., 2008). The BTH-induced resistance have been shown to involve the activation of SAR mechanisms, based on salycilic acid pathway (Friedrich et al., 1996) with consequent up-regulation of defence genes (Bovie et al., 2004) and

accumulation of phenolic compounds (Iriti et al., 2004). In grapevine, BTH significantly reduced downy mildew symptoms both locally and systemically without any direct toxic effect on *P. viticola* sporangia, indicating that the control mechanism is related to resistance induction in the plant (Perazzolli et al., 2008). Although application of BTH directly induces the expression of defence-related genes, a strong reduction of plant growth is correlated to repetitive applications of this resistance inducer (Perazzolli et al., 2008). For this reason it has been proposed that BTH cause significant energy costs for the allocation of resources into defence process but away from plant growth (van Hulten et al., 2006).

Trichoderma spp. (class *Ascomycota*, ord. *Hypocreales*, fam. *Hypocreaceae*) are commonly found in the soil and they have been initially studied for their ability to interact with soil pathogens through mycoparasitism, production of antibiotics, competition for nutrients in the rhizosphere (Shoresh et al., 2010). Moreover, pathogen control occurs also through the ability of the fungi to reprogram plant gene expression that, as a consequence, give to the plant the induced systemic resistance (ISR) (Shoresh et al., 2010). The *Trichoderma harzianum* T39 strain was initially characterized for the control of *Botrytis cinerea* (Elad 1994) and it has been shown to activate a JA/ET-dependent ISR in *Arabidopsis* (Korolev et al., 2008). Application of T39 has been shown to reduce downy mildew symptoms in grapevine by activating ISR (Perazzolli et al., 2008) without apparent energy cost for the plant (Perazzolli et al., 2011). Particularly, the reduction of downy mildew symptoms is related to a dual effect: direct modulation of defence genes and activation of a priming state (Perazzolli et al., 2011). T39-induced resistance partially inhibits some disease-related processes and specifically activates some defence processes known to be implicated in the reaction of resistant genotypes to downy mildew (Perazzolli et al., 2012). Moreover, histological analyses indicate that T39 primes grapevine defences through the accumulation of callose in stomata guard cells and through the production of reactive oxygen species (ROS) in leaf tissue (Palmieri et al., 2012).

Resistance inducers in commercial field conditions: applications and limitations

Induced resistance offers a broad-spectrum disease control based on plant’s own defences (Vallad and Goodman, 2004; van der Ent et al., 2009; Walters, 2009), and it may represent a promising biocontrol method for crop diseases (Vallad and Goodman, 2004). However, the potential of induced resistance is yet to be realized mainly because of its inconsistent efficacy

under field conditions (Walters, 2009; Walters et al., 2012; Walters and Boyle, 2005), suggesting a lack of knowledge on the mechanisms underlying resistance activation in plants. Induced resistance is a plant-mediated mechanism, and its expression under field conditions is likely to be influenced by a number of factors, including environment, genotype and crop nutrition (Walters et al., 2013). Thus, besides optimal treatment timing and integration with other protection programme (Walters et al., 2012), the influence of the plant genotype and of environmental stresses on the induced resistance should be clarified for each crop, in order to maximize the efficacy of this biocontrol method (Walters, 2009; Walters et al., 2012; Walters and Boyle, 2005). Activation of defense processes has been found to be negatively affected by limiting nitrogen supply (Dietrich et al., 2004; Walters et al., 2005), and it was found to be variable depending on the plant genotype (Córdova-Campos et al., 2012; Sharma et al., 2010; Tucci et al., 2011; Walters et al., 2011). Concerns about the impact of abiotic stresses on agriculture have been raised in the last decade, especially in light of the predicted effects of climate changes (Lobell et al., 2011). High temperatures and drought associated with heat waves may occur with increased frequency as a result of climate change (IPCC 2007), threatening crop production and influencing interactions with both pathogenic (Eastburn et al., 2011; Pautasso et al., 2012; Yasuda et al., 2008) and beneficial (Compant et al., 2010) microorganisms. Whereas the effects of abiotic stresses on plant resistance to pathogens have been extensively studied (Atkinson and Urwin 2012), their effects on the induced resistance mechanism has received little attention (Baysal et al., 2007). Moreover, the effects of increased temperature on beneficial plant-associated microorganisms have been found to be variable (Compant et al., 2010), highlighting the complexity of this system. A deeper understanding of the expression of the induced resistance in plants exposed to abiotic stresses and in different varieties could help to understand the inconsistency of efficacy in field conditions and to establish strategies to optimize the use of resistance inducers (Walters et al., 2005).

The present work

This PhD thesis have been supported by the EnviroChance Project (www.envirochange.eu), funded by the autonomous Province of Trento. The project focuses on global change and sustainable management of agriculture in highly developed mountain

environment. It aims at assessing the short-term biological, environmental and economic impact of climatic change on agriculture at the regional level (Trentino) particularly on quality and pest management that are more likely to be influenced by climate change in the short term.

Grapevine is one of the most important crops in Trentino-Alto Adige (northern Italy). Downy mildew, caused by the obligate biotrophic Oomycete *P. viticola* is an important disease because of the presence of pathogen inoculum and favourable environmental conditions for its development. Uncontrolled epidemics of *P. viticola* may result in yield losses, as well as a reduction in the quality of the produced wine (Gessler et al., 2011). Control of the diseases relies mainly on the use of chemical fungicides. The risk of epidemic infections and management control strategies is based on prediction models that, however, fail to prevent disease development (Rumbou and Gessler 2004). Alternative methods for controlling downy mildew have been studied, including the use of microbial biocontrol agents (Gessler et al., 2011). The *Trichoderma harzianum* T39 strain (T39) is a biocontrol organism effective in reducing downy mildew symptoms through a plant mediated resistance mechanism (Perazzolli et al., 2008). However, the efficacy of resistance inducers is variable and often inconsistent when used in field condition (Walters et al., 2013). The future climate change will likely influence the efficacy of the resistance inducers on plants against downy mildew disease, thus, the thesis has two principal objectives. First objective was to un-reveal evidences supporting the reason for predominance of particular genotypes during the epidemic season. The investigation of the mechanisms behind the selection of predominant genotypes during an epidemic may help in understanding the strategies on how these genotypes spread during an epidemic. The prevention of the establishment of predominant genotypes could improve the actual control measures used for the control of downy mildew of grapevine. The second objective was to investigate the effect of abiotic stresses and of different grapevine cultivars on the level of induced resistance against downy mildew. Analysis of these variables on the induced resistance may help to elucidate possible interfering factors to the real efficacy of the resistance inducers, thus to track the way for the improvement of biocontrol agents to be used for the appropriate climatic condition and for the most responsive cultivars.

To achieve this, the work was structured in the following order:

in chapter 2 I evaluated i) differences in latency in *P. viticola* genotypes deriving from different climatic regions at non-optimal conditions and ii) polycyclic infections of *P. viticola* isolates in a competitive environment by co-inoculating genotypes with similar aggressiveness components, measured considering the latent period, infection frequency and spore production of pathogen's life cycle (Corio-Costet et al., 2011). Competition between the genotypes was assessed and optimized using a quantification method based on microsatellite marker developed for *P. viticola* (Delmotte et al., 2006; Gobbin et al., 2003).

In chapter 3 I investigated the effect of short exposure to heat and drought stresses on the level of resistance activated by T39 against downy mildew in grapevine. Molecular events in the T39-induced resistance under heat and drought stresses were also investigated by analysing the biomarkers known to be modulated during this defence process (Perazzolli et al., 2012). The ultimate goal is to be able to predict the effect of heat and drought on the efficacy of T39-induced resistance under field conditions, especially in a view of predicted climate change.

In chapter 4 we characterized the effect of grapevine genotypes on the T39-induced resistance compared to the BTH activated resistance against downy mildew. The induced resistance was also investigated analysing biomarkers known to be modulated during these defence processes (Perazzolli et al., 2012). The objective was the evaluation of the induced resistance in different cultivars, in order to improve the use of BTH and T39 for the most appropriate cultivars and to highlight the necessity to find other microbial resistance inducers for the less responsive cultivars.

REFERENCES

Agrios, G. N. (1997) The downy mildews. In: Plant Pathol., 4rth eds, Academic Press, San Diego, CA., 278-283

Allègre, M., Héloir, M. C., Trouvelot, S., Daire, X., Pugin, A., Wendehenne, D. and Adrian, M. (2009). Are grapevine stomata involved in the elicitor-induced protection against downy mildew? Mol. Plant-Microbe Interact. 8: 977-986

Atkinson, N. J. and Urwin, P. E. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. J. Exp. Bot. 10: 3523-3543

Aziz, A., Poinssot, B., Daire, X., Adrian, M., Bézier, A., Lambert, B., Joubert, J.-M. and Pugin, A. (2003). Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopara viticola*. Mol. Plant-Microbe Interact. 12: 1118-1128

Aziz, A., Trotel-Aziz, P., Dhuicq, L., Jeandet, P., Couderchet, M. and Vernet, G. (2006). Chitosan oligomers and copper sulfate induce grapevine defense reactions and resistance to gray mold and downy mildew. Phytopathology. 11: 1188-1194

Baysal, Ö., Gürsoy, Y. Z., Örnek, H., Çetinel, B. and Teixeira da Silva, J. A. (2007). Enhanced systemic resistance to bacterial speck disease caused by *Pseudomonas syringae* pv. tomato by dl-β-aminobutyric acid under salt stress. Physiol. Plant. 3: 493-506

Bellin, D., Peressotti, E., Merdinoglu, D., Wiedemann-Merdinoglu, S., Adam-Blondon, A.-F., Cipriani, G., Morgante, M., Testolin, R. and Di Gaspero, G. (2009). Resistance to *Plasmopara viticola* in grapevine 'Bianca' is controlled by a major dominant gene causing localised necrosis at the infection site. TAG Theoretical and Applied Genetics. 1: 163-176

Boller, T. and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu. Rev. Plant Biol. 1: 379-406

Borie, B., Jeandet, P., Parize, A., Bessis, R. and Adrian, M. (2004). Resveratrol and stilbene synthase mRNA production in grapevine leaves treated with biotic and abiotic phytoalexin elicitors. American journal of enology and viticulture. 1: 60-64

Bovie, C., Ongena, M., Thonart, P. and Dommes, J. (2004). Cloning and expression analysis of cDNAs corresponding to genes activated in cucumber showing systemic acquired resistance after BTH treatment. BMC Plant Biol. 1: 15

Compant, S., Van Der Heijden, M. G. A. and Sessitsch, A. (2010). Climate change effects on beneficial plant–microorganism interactions. FEMS Microbiol. Ecol. 2: 197-214

Conrath, U., Beckers, G. J. M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M.-A., Pieterse, C. M. J., Poinssot, B., Pozo, M. J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L. and Mauch-Mani, B. (2006). Priming: getting ready for battle. Mol. Plant-Microbe Interact. 10: 1062-1071

Córdova-Campos, O., Adame-Álvarez, R., Acosta-Gallegos, J. and Heil, M. (2012). Domestication affected the basal and induced disease resistance in common bean (*Phaseolus vulgaris*). Eur. J. Plant Pathol. 2: 367-379

Corio-Costet, M.-F., Dufour, M.-C., Cigna, J., Abadie, P. and Chen, W.-J. (2011). Diversity and fitness of *Plasmopara viticola* isolates resistant to QoI fungicides. Eur. J. Plant Pathol. 2: 315-329

Dai, G. H., Andary, C., Mondolot-Cosson, L. and Boubals, D. (1995). Histochemical studies on the interaction between three species of grapevine, *Vitis vinifera*, *V. rupestris* and *V.*

rotundifolia and the downy mildew fungus, *Plasmopara viticola*. Physiol. Mol. Plant Pathol. 3: 177-188

Delmotte, F., Chen, W. J., Richard-Cervera, S., Greif, C., Papura, D., Giresse, X., Mondor-Genson, G. and Corio-Costet, M. F. (2006). Microsatellite DNA markers for *Plasmopara viticola*, the causal agent of downy mildew of grapes. Mol. Ecol. Notes. 2: 379-381

Dercks, W. and Creasy, L. L. (1989). Influence of fosetyl-Al on phytoalexin accumulation in the *Plasmopara viticola*-grapevine interaction. Physiol. Mol. Plant Pathol. 3: 203-213

Di Gaspero, G., Cipriani, G., Adam-Blondon, A. F. and Testolin, R. (2007). Linkage maps of grapevine displaying the chromosomal locations of 420 microsatellite markers and 82 markers for R-gene candidates. Theor. Appl. Genet. 7: 1249-1263

Dietrich, R., Ploß, K. and Heil, M. (2004). Constitutive and induced resistance to pathogens in *Arabidopsis thaliana* depends on nitrogen supply. Plant Cell Environ. 7: 896-906

Dìez-Navajas, A. M., Wiedemann-Merdinoglu, S., Greif, C. and Merdinoglu, D. (2008). Nonhost versus host resistance to the grapevine downy mildew, *Plasmopara viticola*, studied at the tissue level. Phytopathology. 7: 776-780

Dubreuil-Maurizi, C., Trouvelot, S., Frettinger, P., Pugin, A., Wendehenne, D. and Poinssot, B. (2010). β-aminobutyric acid primes an NADPH oxidase–dependent reactive oxygen species production during grapevine-triggered immunity. Mol. Plant-Microbe Interact. 8: 1012-1021

Eastburn, D. M., McElrone, A. J. and Bilgin, D. D. (2011). Influence of atmospheric and climatic change on plant–pathogen interactions. Plant Pathol. 1: 54-69

Elad, Y. (1994). Biological control of grape grey mould by *Trichoderma harzianum*. Crop Protect. 1: 35-38

Ferri, M., Tassoni, A., Franceschetti, M., Righetti, L., Naldrett, M. J. and Bagni, N. (2009). Chitosan treatment induces changes of protein expression profile and stilbene distribution in *Vitis vinifera* cell suspensions. Proteomics. 3: 610-624

Figueiredo, A., Fortes, A. M., Ferreira, S., Sebastiana, M., Choi, Y. H., Sousa, L., Acioli-Santos, B., Pessoa, F., Verpoorte, R. and Pais, M. S. (2008). Transcriptional and metabolic profiling of grape (*Vitis vinifera L.*) leaves unravel possible innate resistance against pathogenic fungi. J. Exp. Bot. 12: 3371-3381

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M. G., Meier, B., Dincher, S., Staub, T., Uknes, S., Métraux, J.-P., Kessmann, H. and Ryals, J. (1996). A benzothiadiazole derivative induces systemic acquired resistance in tobacco. The Plant Journal. 1: 61-70

Gessler, C., Pertot, I. and Perazzolli, M. (2011). *Plasmopara viticola* : a review of knowledge on downy mildew of grapevine and effective disease management. Phytopathol. Mediterr. 3-44

Gindro, K., Pezet, R. and Viret, O. (2003). Histological study of the responses of two *Vitis vinifera* cultivars (resistant and susceptible) to *Plasmopara viticola* infections. Plant Physiol. Biochem. 9: 846-853

Gobbin, D., Bleyer, G., Keil, S., Kassemeyer, H. H. and Gessler, C. (2007). Evidence for sporangial dispersal leading to a single infection event and a sudden high incidence of grapevine downy mildew. Plant Pathol. 5: 843-847

Gobbin, D., Jermini, M., Loskill, B., Pertot, I., Raynal, M. and Gessler, C. (2005). Importance of secondary inoculum of *Plasmopara viticola* to epidemics of grapevine downy mildew. Plant Pathol. 4: 522-534

Gobbin, D., Pertot, I. and Gessler, C. (2003). Identification of microsatellite markers for *Plasmopara viticola* and establishment of high throughput method for SSR snalysis. Eur. J. Plant Pathol. 2: 153-164

Gobbin, D., Pertot, I. and Gessler, C. (2003). Genetic structure of a *Plasmopara viticola* population in an isolated italian mountain vineyard. J. Phytopathol. 11-12: 636-646

Gobbin, D., Rumbou, A., Linde, C. C. and Gessler, C. (2006). Population genetic structure of *Plasmopara viticola* after 125 years of colonization in European vineyards. Mol. Plant Pathol. 6: 519-531

Godard, S., Slacanin, I., Viret, O. and Gindro, K. (2009). Induction of defence mechanisms in grapevine leaves by emodin- and anthraquinone-rich plant extracts and their conferred resistance to downy mildew. Plant Physiol. Biochem. 9: 827-837

Hamiduzzaman, M., Jakab, G., Barnavon, L., Neuhaus, J. and Mauch-Mani, B. (2005). beta-aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. Mol. Plant-Microbe Interact. 819 - 829

IPCC Intergovernmental Panel on Climate Change 2007. Synthesis report. Summary for policymakers. Avalable at http://www.ipcc.ch.

Harm, A., Kassemeyer, H.-H., Seibicke, T. and Regner, F. (2011). Evaluation of chemical and natural resistance inducers against downy mildew (*Plasmopara viticola*) in grapevine. American journal of enology and viticulture. 2: 184-192

Iriti, M., Rossoni, M., Borgo, M. and Faoro, F. (2004). Benzothiadiazole enhances resveratrol and anthocyanin biosynthesis in grapevine, meanwhile improving resistance to *Botrytis cinerea*. J. Agric. Food Chem. 14: 4406-4413

Jürges, G., Kassemeyer, H. H., Dürrenberger, M., Düggelin, M. and Nick, P. (2009). The mode of interaction between *Vitis* and *Plasmopara viticola* Berk. & Curt. Ex de Bary depends on the host species. Plant Biol. 6: 886-898

Korolev, N., Rav David, D. and Elad, Y. (2008). The role of phytohormones in basal resistance and *Trichoderma*-induced systemic resistance to *Botrytis cinerea* in *Arabidopsis thaliana*. BioControl. 4: 667-683

Kortekamp, A. (2006). Expression analysis of defence-related genes in grapevine leaves after inoculation with a host and a non-host pathogen. Plant Physiol. Biochem. 1: 58-67

Kortekamp, A. and Zyprian, E. (2003). Characterization of *Plasmopara* resistance in grapevine using in vitro plants. J. Plant Physiol. 1393 - 1400

Lobell, D. B., Schlenker, W. and Costa-Roberts, J. (2011). Climate trends and global crop production since 1980. Science. 6042: 616-620

Matasci, C., Jermini, M., Gobbin, D. and Gessler, C. (2010). Microsatellite based population structure of *Plasmopara viticola* at single vine scale. Eur. J. Plant Pathol. 4: 501-508

Moreira, F., Madini, A., Marino, R., Zulini, L., Stefanini, M., Velasco, R., Kozma, P. and Grando, M. S. (2011). Genetic linkage maps of two interspecific grape crosses (*Vitis* spp.) used to localize quantitative trait loci for downy mildew resistance. Tree Genet. Genom. 1: 153-167

Palmieri, M. C., Perazzolli, M., Matafora, V., Moretto, M., Bachi, A. and Pertot, I. (2012). Proteomic analysis of grapevine resistance induced by *Trichoderma harzianum* T39 reveals specific defence pathways activated against downy mildew. J. Exp. Bot. 17: 6237-6251

Pariaud, B., Ravigné, V., Halkett, F., Goyeau, H., Carlier, J. and Lannou, C. (2009). Aggressiveness and its role in the adaptation of plant pathogens. Plant Pathol. 3: 409-424

Pautasso, M., Döring, T., Garbelotto, M., Pellis, L. and Jeger, M. (2012). Impacts of climate change on plant diseases—opinions and trends. Eur. J. Plant Pathol. 1: 295-313

Perazzolli, M., Dagostin, S., Ferrari, A., Elad, Y. and Pertot, I. (2008). Induction of systemic resistance against *Plasmopara viticola* in grapevine by *Trichoderma harzianum* T39 and benzothiadiazole. Biol. Control. 2: 228-234

Perazzolli, M., Moretto, M., Fontana, P., Ferrarini, A., Velasco, R., Moser, C., Delledonne, M. and Pertot, I. (2012). Downy mildew resistance induced by *Trichoderma harzianum* T39 in susceptible grapevines partially mimics transcriptional changes of resistant genotypes. BMC Genomics. 1: 660

Perazzolli, M., Roatti, B., Bozza, E. and Pertot, I. (2011). *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defense without costs for grapevine. Biol. Control. 1: 74-82

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S. and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. Nat. Chem. Biol. 5: 308-316

Pieterse, C. M. J., Van Pelt, J. A., Ton, J., Parchmann, S., Mueller, M. J., Buchala, A. J., Métraux, J.-P. and Van Loon, L. C. (2000). Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. Physiol. Mol. Plant Pathol. 3: 123-134

Polesani, M., Bortesi, L., Ferrarini, A., Zamboni, A., Fasoli, M., Zadra, C., Lovato, A., Pezzotti, M., Delledonne, M. and Polverari, A. (2010). General and species-specific transcriptional responses to downy mildew infection in a susceptible (*Vitis vinifera*) and a resistant (*V. riparia*) grapevine species. BMC Genomics. 1: 117

Polesani, M., Desario, F., Ferrarini, A., Zamboni, A., Pezzotti, M., Kortekamp, A. and Polverari, A. (2008). cDNA-AFLP analysis of plant and pathogen genes expressed in grapevine infected with *Plasmopara viticola*. BMC Genomics. 1: 142

Reuveni, M. (2001). Activity of trifloxystrobin against powdery and downy mildew diseases of grapevines. Can. J. Plant Pathol. 1: 52-59

Rumbou, A. and Gessler, C. (2004). Genetic dissection of *Plasmopara viticola* population from a Greek vineyard in two consecutive years. Eur. J. Plant Pathol. 4: 379-392

Rumbou, A. and Gessler, C. (2006). Particular structure of *Plasmopara viticola* populations evolved under Greek island conditions. Phytopathology. 5: 501-509

Sharma, K., Butz, A. F. and Finckh, M. R. (2010). Effects of host and pathogen genotypes on inducibility of resistance in tomato (*Solanum lycopersicum*) to *Phytophthora infestans*. Plant Pathol. 6: 1062-1071

Shoresh, M., Harman, G. E. and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. Annu. Rev. Phytopathol. 1: 21-43

Slaughter, A., Hamiduzzaman, M., Gindro, K., Neuhaus, J.-M. and Mauch-Mani, B. (2008). Beta-aminobutyric acid-induced resistance in grapevine against downy mildew: involvement of pterostilbene. Eur. J. Plant Pathol. 1: 185-195

Thuerig, B., Binder, A., Boller, T., Guyer, U., Jiménez, S., Rentsch, C. and Tamm, L. (2006). An aqueous extract of the dry mycelium of *Penicillium chrysogenum* induces resistance in several crops under controlled and field conditions. Eur. J. Plant Pathol. 2: 185-197

Thuerig, B., Slaughter, A., Marouf, E., Held, M., Mauch-Mani, B. and Tamm, L. (2011). Site-specific field resistance of grapevine to *Plasmopara viticola* correlates to altered gene

expression and was not modulated by the application of organic amendments. Eur. J. Plant Pathol. 2: 255-265

Trouvelot, S., Varnier, A. L., Allègre, M., Mercier, L., Baillieul, F., Arnould, C., Gianinazzi-Pearson, V., Klarzynski, O., Joubert, J. M., Pugin, A. and Daire, X. (2008). A β-1,3 glucan sulfate induces resistance in grapevine against *Plasmopara viticola* through priming of defense responses, including HR-like cell death. Mol. Plant-Microbe Interact. 2: 232-243

Tucci, M., Ruocco, M., De Masi, L., De Palma, M. and Lorito, M. (2011). The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. Mol. Plant Pathol. 4: 341-354

Unger, S., Buche, C., Boso, S. and Kassemeyer, H.-H. (2007). The course of colonization of two different *Vitis* genotypes by *Plasmopara viticola* indicates compatible and incompatible host-pathogen interactions. Phytopathology. 7: 780-786

Vallad, G. E. and Goodman, R. M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. Crop Sci. 6: 1920-1934

van der Ent, S., Koornneef, A., Ton, J. and Pieterse, C. M. J. (2009) Induced Resistance – Orchestrating Defence Mechanisms through Crosstalk and Priming. In: Annual Plant Reviews Volume 34: Molecular Aspects of Plant Disease Resistance, Wiley-Blackwell, 334-370

van Hulten, M., Pelser, M., van Loon, L. C., Pieterse, C. M. J. and Ton, J. (2006). Costs and benefits of priming for defense in *Arabidopsis*. Proceedings of the National Academy of Sciences. 14: 5602-5607

Verhagen, B. W. M., Glazebrook, J., Zhu, T., Chang, H.-S., van Loon, L. C. and Pieterse, C. M. J. (2004). The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. Mol. Plant-Microbe Interact. 8: 895-908

Walters, D. and Heil, M. (2007). Costs and trade-offs associated with induced resistance. Physiol. Mol. Plant Pathol. 1–3: 3-17

Walters, D. R. and Boyle, C. (2005). Induced resistance and allocation costs: What is the impact of pathogen challenge? Physiol. Mol. Plant Pathol. 1-2: 40-44

Walters, D., Walsh, D., Newton, A. and Lyon, G. (2005). Induced resistance for plant disease control: maximizing the efficacy of resistance elicitors. Phytopathology. 12: 1368-1373

Walters, D. R. (2009). Are plants in the field already induced? Implications for practical disease control. Crop Protect. 6: 459-465

Walters, D. R., Havis, N. D., Paterson, L., Taylor, J. and Walsh, D. J. (2011). Cultivar effects on the expression of induced resistance in spring barley. Plant Dis. 5: 595-600

Walters, D. R., Avrova, A., Bingham, I. J., Burnett, F. J., Fountaine, J., Havis, N. D., Hoad, S. P., Hughes, G., Looseley, M., Oxley, S. J. P., Renwick, A., Topp, C. F. E. and Newton, A. C. (2012). Control of foliar diseases in barley: towards an integrated approach. Eur. J. Plant Pathol. 1: 33-73

Walters, D. R., Ratsep, J. and Havis, N. D. (2013). Controlling crop diseases using induced resistance: challenges for the future. J. Exp. Bot.

Wong, F. P., Burr, H. N. and Wilcox, W. F. (2001). Heterothallism in *Plasmopara viticola*. Plant Pathol. 4: 427-432

Yasuda, M., Ishikawa, A., Jikumaru, Y., Seki, M., Umezawa, T., Asami, T., Maruyama-Nakashita, A., Kudo, T., Shinozaki, K., Yoshida, S. and Nakashita, H. (2008).

Antagonistic interaction between systemic acquired resistance and the abscisic acid–mediated abiotic stress response in *Arabidopsis*. Plant Cell. 6: 1678-1692

Young, G. K., Cooke, L. R., Kirk, W. W., Tumbalam, P., Perez, F. M. and Deahl, K. L. (2009). Influence of competition and host plant resistance on selection in *Phytophthora infestans* populations in Michigan, USA and in Northern Ireland. Plant Pathol. 4: 703-714

Chapter 2

Co-inoculated *P. viticola* genotypes compete for the infection of the host independently from the aggressiveness components

Benedetta Roatti, Cesare Gessler, Michele Perazzolli, Ilaria Pertot

Accepted for publication on January 7th (2013) in European Journal of Plant Pathology, June 2013, Volume 136, Issue 2, pp 363-371.

ABSTRACT

During *Plasmopara viticola* epidemics only few genotypes produce most of the secondary lesions and dominate in the population. Selection of dominant genotypes is hypothesized to be linked to environmental conditions and can occur rapidly, particularly if there is also difference between genotypes in terms of fitness and aggressiveness. Measurements of aggressiveness components can largely determine the rate of an epidemic development, although the components of aggressiveness do not take into account potential direct competition between genotypes. Differences in aggressiveness have been also reported to be greater under non-optimal conditions suggesting for genotypes adaptation to different conditions. To evaluate differences in latency at non-optimal conditions, we characterized genotypes deriving from different climatic regions at three different temperatures (15, 25 and 35°C) and we found no differences. To investigate whether other factors may impact on competition between *P. viticola* genotypes, we evaluated polycyclic infections of *P. viticola* by co-inoculating three genotypes with similar aggressiveness components in two different co-inoculation experiments and an increasing prevalence of one of the two genotypes was observed. Competition was not related to the origin of the genotype and we hypothesize that competitive selection is modulated by differences in the secretion of effector molecules which can contribute to the establishment of dominant genotypes over an epidemic season.

INTRODUCTION

Downy mildew is one of the most damaging fungal diseases of grapevine (*Vitis vinifera*) worldwide. The causal agent, *Plasmopara viticola* (Berck. and Curt.) Berl. and de Toni, is a heterothallic (Wong et al., 2001) diploid oomycete and obligate biotrophic parasite native to North America. The pathogen overwinters as sexually-produced oospores in fallen leaves and the disease cycle starts when the oospores germinate producing sporangia and zoospores, which cause the primary infections (Gessler et al., 2011). All green parts of the plants which have active stomata can be attacked, and symptoms are easily recognized as yellowish lesions on leaves, known as “oilspots”. When conditions are favorable, microsporangia containing asexually-produced zoospores are released from the primary lesion and asexual secondary infection cycles occur throughout the growing season alongside primary infections (Gessler et al., 2011).

Population genetic studies with microsatellite markers (SSR) have focused specifically on characterizing population structures in the field on large and fine spatial scales. These studies have shown that *P. viticola* has high evolutionary potential due to large population sizes, high genotypic diversity, moderate migration ability of asexual propagules, a mixed reproductive system and random mating (Gobbin et al., 2006). Primary oosporic infections have been shown to contribute in highly varying degrees to an epidemic throughout the season, and despite producing large amounts of sporangia (Reuveni 2003), only a few of these genotypes have been found to produce secondary lesions and to predominate in summer populations of the pathogen (Gobbin et al., 2007; Gobbin et al., 2005; Gobbin et al., 2006; Rumbou and Gessler 2004; Rumbou and Gessler 2006). The main reason the majority of genotypes fail to reproduce further after primary infection has been hypothesized as being linked to variable sensitivity to environmental conditions (Gobbin et al., 2005). Moreover, selection of dominant genotypes within populations can occur rapidly, particularly if there is a considerable difference between genotypes in terms of their fitness (Young et al., 2009), defined as the ability of a phenotype to contribute to the next generation (Antonovics and Alexander 1989). In the interaction between plant and pathogen genotypes, single generation measurements, such as infection efficacy, latent period, spore production rate, infectious period and lesion size are commonly used as “quantitative traits related to pathogenicity” which determine the aggressiveness of single

isolates (Pariaud et al., 2009). Many studies have documented differences in aggressiveness among isolates belonging to the same pathotype (Pariaud et al., 2009) and, in experiments of co-inoculation of different *P. viticola* isolates, changes in their frequency may be largely dependent on differences in the aggressiveness components measured for the single isolates (Corio-Costet et al., 2011). However, while the aggressiveness of individuals may affect the competition between them and could explain the selection of dominant genotypes in field, it is also possible that direct competition or other mechanisms between genotypes also plays a role(Young et al., 2009). Among pathogen isolates, differences in aggressiveness have also been reported to be greater under non-optimal conditions (Pariaud et al., 2009), suggesting that differential responses in terms of aggressiveness may exist between genotypes originating from different climatic regions where genotypes may have been adapted to growing at different conditions.

To study differences in growth among *P. viticola* isolates at non-optimal conditions and to evaluate whether factors other than aggressiveness components could be responsible for genotype selection, we evaluated i) differences in latency in *P. viticola* genotypes deriving from different climatic regions at non-optimal conditions and ii) polycyclic infections of *P. viticola* in a competitive environment by co-inoculating genotypes with similar aggressiveness components, measured considering the latent period, infection frequency and spore production of pathogen's life cycle (Corio-Costet et al., 2011). Competition between the genotypes was assessed using a method based on microsatellite marker developed for *P. viticola* (Delmotte et al., 2006; Gobbin et al., 2003). Moreover, the microsatellite-based quantification method (Naef et al., 2006; Reininger et al., 2011) was optimized for quick detection and quantification of *P. viticola* genotypes at different stages of the asexual infection cycle.

MATERIALS AND METHODS

Collection and propagation of *Plasmopara viticola* isolates

Entire leaves or fragments with single *P. viticola* lesions were collected. Each single lesion presumably represents a single *P. viticola* genotype(Gobbin et al., 2003). Vegetal material was placed separately, abaxial side up, on wet blotting paper in Petri dishes and incubated overnight in the dark at 25°C to induce sporulation. Sporangia produced by the lesions were harvested separately in 4 mL of cold (4°C) distilled water and the suspensions were used to

inoculate *Vitis vinifera* cv. Pinot Noir cuttings (rootstock Kober 5BB) by spraying the abaxial leaf surface. Plants were incubated overnight in the dark for 12 hours at 25°C with 99-100% RH, and then maintained at 25°C, 60±10% RH. Once oilspot symptoms appeared, sporulation was induced by placing the plants in the dark overnight at 25°C and 99-100% RH (Perazzolli et al., 2011). The amount of inoculum was increased over four consecutive infection cycles. Once infected leaves reached 80-100% disease severity (i.e. percentage of leaf area covered by sporulation), part of them were harvested, immediately frozen in liquid nitrogen and kept at -80°C for DNA extraction, while the remaining leaves were kept at -20°C for long term storage of the isolates (Laviola et al., 2006).

Effect of temperature on latency in *Plasmopara viticola* genotypes from different regions

Six strains were randomly isolated from single leaf lesions originating from different climatic regions in 2009: two isolates were from northern Italy (Lat: 45.918378, Long: 11.071558), one from central Italy (Lat: 44.486326, Long: 11.333106) two from southern Italy (Lat: 41.136262, Long: 16.871567) and one from Israel (Lat: 31.741015, Long: 35.182514). Sporangia from each lesion were collected and propagated as described above. Leaves from the fourth to the sixth node starting from the apical meristem of 10 weeks old plants grown in greenhouse were detached so that all leaves were of similar age and size. Leaves were randomly placed abaxial side up on moistened filter paper in Petri dishes. Nine leaves per strain were inoculated with 6 droplets of 20 µl of 5 × 104 sporangia mL-1 and incubated in greenhouse under the conditions described above. The day after inoculation, leaves were divided into three groups and incubated in the greenhouse under different temperature conditions: two groups were incubated at 15°C and 25°C, respectively, until sporulation, while the third group was incubated at 35°C for three days, then at 25°C until sporulation. Sporangia appearance was monitored daily through visual notation to estimate the latent period, defined as the time (days) from inoculation to first sporulation.

Microsatellite SSR analysis of *Plasmopara viticola* isolates

DNA was extracted from 100 mg of infected leaves or from 10 mg of freeze-dried inoculum suspensions using the DNeasy plant mini kit (Qiagen, Hilden, Germany). Samples were amplified using primers targeting the polymorphic *P. viticola* SSR loci ISAnew,

BERnew(Gobbin et al., 2003; Matasci et al., 2010), Pv7, Pv13, Pv14, Pv17(Delmotte et al., 2006), where each forward primer was labeled with the fluorophore VIC (Applied Biosystems, Foster City, CA). PCR reactions were carried out with a T-Professional Thermocycler (Biometra, Goettingen, GE) using the DreamTaq DNA Polymerase (Fermentas, St. Leon-Rot, Germany). PCR product was diluted 1:10 with sterile water, and 0.5 µL was mixed with 9.2 µL of HiDi formamide and 0.2 µL of GeneScan 500 LIZ size standard (Applied Biosystems). Samples were denatured for 2 minutes at 94°C and cooled on ice. Fragments were separated on an ABI PRISM 3130 sequencer (Applied Biosystems) following the manufacturer's instructions, and analyzed using the Genemapper v 4.0 software (Applied Biosystems). PCR reactions and fragment analysis were performed in triplicate.

Aggressiveness components and fitness index

Three genotypes named BO, VOL 1 and VOL 2 were selected based on SSR profiles and analyzed for latent period, infection frequency and spore production using the methods described by Corio-Costet et al. (2011). All experiments were carried out as described above however using leaf disks with diameter of 2 cm instead of whole leaves. Three replicates (plates) were used for each genotype. Each disk was inoculated with three droplets (15 µL) of 2.5×10^3 sporangia mL-1 on the abaxial side and incubated in greenhouse at 25° C. Sporangia appearance was monitored daily through visual notation to estimate the latent period (LP), defined as the time (days) from inoculation to first sporulation. Sporangia production was calculated 7 days after the inoculum as the total number of sporangia produced per plate (N_t) divided by the 562 deposited sporangia (N_{t0}) (Corio-Costet et al., 2011). Sporulating spots of all leaf disks of each Petri dish were suspended in 20 µL of water and the number of sporangia per plate was determined using a hemocytometer. Infection frequency (IF) was calculated as the proportion of inoculated spots per plate (Corio-Costet et al., 2011) on which lesions had developed seven days post-infection. The fitness index (FI) was calculated for each isolate using the formula described by Corio-Costet et al. (2011) $FI = Ln (N_t/N_{t0} \times IF \times 1/LP)$. Experiments were repeated twice independently.

Co-inoculations with two different *Plasmopara viticola* genotypes

Fresh sporangia deriving from the three selected isolates were used to prepare inoculum suspensions. Two co-inoculations experiments were performed combining the different genotypes in two different couples (BO and VOL 1; VOL 1 and VOL 2). Leaves were washed in cold (4°C) distilled water and sporangia concentration was adjusted to 10^5 sporangia mL^{-1}. Inoculum suspensions were prepared by mixing the genotypes in varying proportions and used to inoculate *V. vinifera* cv. Pinot Noir cuttings at the conditions previously described. In the first co-inoculation experiment (BO and VOL 1), genotype BO was inoculated at 10, 30, 50, 70 and 80% of the total infection while in the second co-inoculation experiment (VOL 1 and VOL 2) genotype VOL 2 was inoculated at 30, 50 and 80% of the total infection. Each inoculum suspension was sprayed onto three leaves of single plants and they were considered as repetitions in the experiment. Leaves were selected as previously described and approximately 2 mL of inoculum suspension per leaf was sprayed. As controls, plants were inoculated separately with genotypes alone. Residual inoculum suspensions of first inoculum solution (around 10 mL) were freeze-dried and used for DNA extraction as described above. Plants were maintained in greenhouse in the conditions previously described until all leaves showed symptoms. Infected leaves were then detached, individually transferred onto wet blotting paper in Petri dishes, and incubated overnight in the dark to induce sporulation. Disease severity was assessed in control plants as percentage of abaxial leaf area covered by sporulation (Perazzolli et al., 2011). Sporangia from each leaf were harvested separately in 4 mL of cold distilled water and each sporangia solution was uniquely used to inoculate one single leaf on a new plant. The leaves after washing off the sporangia were immediately frozen in liquid nitrogen and kept at -80°C for DNA extraction. The infection procedure was repeated in order to cover three consecutive asexual infection cycles.

Competitive microsatellite PCR and analysis of fluorescence data

The quantification method based on competitive microsatellite PCR relies on co-amplification of microsatellites of different sizes (Naef et al., 2006; Reininger et al., 2011). Competitive PCR with the SSR marker Pv14 (Delmotte et al., 2006) and fragment analysis was performed as described above on DNA mixtures of the genotypes BO (Pv14 allele size: 125) and VOL 1 (Pv14 allele size: 121) and on DNA mixtures of the genotypes VOL 1 (Pv14 allele size:

121) and VOL 2 (Pv14 allele size: 125). Total fluorescence per sample was calculated as the sum of the heights of each peak. BO and VOL 2 normalized fluorescence was calculated as its peak height divided by total fluorescence. Normalized fluorescence values were plotted against the known quantities of genotype DNA and a linear regression analysis was performed on data points.

Statistical analysis

Data were analysed using the Statistica 9 software (StatSoft, Tulsa, OK). Data with normal distribution (*K-S* test, $P < 0.05$) were validated for variance homogeneity (Leven's test, $P < 0.05$) and the analysis of variance (ANOVA) was performed using Fisher's test to detect significant differences between genotypes. In case data were not homogeneous according to *K-S* test (spore production and infection frequency data) data were transformed in LOG_{10} and analysed as described above.

RESULTS

Effect of temperature on latency in *Plasmopara viticola* genotypes from different regions

To evaluate differences in genotypes development we compared the latency of six strains deriving from four climatic regions at three different incubation temperatures. Sporulation occurred in all strains incubated at 25°C five days after inoculation, while those incubated at 15°C and those kept at 35°C displayed sporulation seven days after inoculation, showing that growth rates at different temperatures were independent of the origin of the strains. No difference in latent period was observed among isolates.

Genotype selection and measurement of fitness index

Of the *P. viticola* genotypes collected, three were selected for the following characteristics: different allele size at locus Pv14 for at least one genotype (VOL1, Pv14 allele size 121; BO and VOL2, Pv14 allele size 125) and homozygosis for this specific SSR marker. Given that they were collected in Bologna (Emilia Romagna, Lat: 45.918378, Long: 11.071558) and Volano (Trentino region, Lat: 44.486326, Long: 11.333106) they were named BO, VOL 1 and VOL 2. Sporangia production and infection frequency were found to be similar in all three

genotypes (Fig. 1a and 1b), as was latent period (five days after inoculation at 25 C°). The calculated fitness index (Fig. 1c), which associates the spore production, infection frequency and latency period for all three genotypes, were ranging from mean values of 2.1 (VOL 2), 2.2 (BO) and 2.5 (VOL 1), however without being significantly different according to Fisher's test (*P* > 0.05).

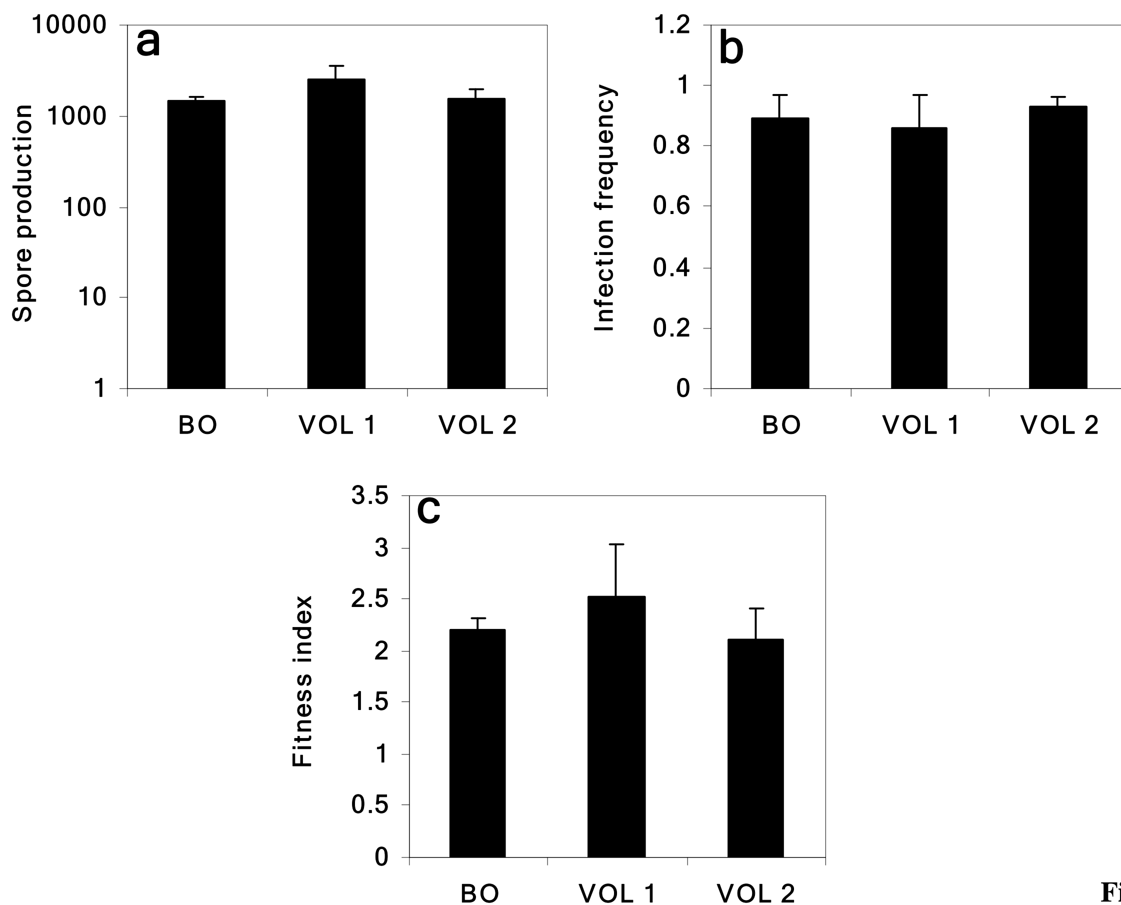

Figure 1. Sporangia production x (c) of *Plasmopara viticola* genotypes BO, VOL 1 and VOL 2. No differences were found between genotypes (Fisher's test, P > 0.05). Mean values and standard errors of three replications of one representative experiment are presented

Optimization of competitive microsatellite PCR for identification and quantification of *Plasmopara viticola* genotypes

Amplification with the SSR Pv14 of the DNA of the two isolates BO (allele size 125) and VOL1 (allele size 121) mixed in 1:1 ratio yielded two clearly distinct peaks with the same height (Fig. 2), showing that the two alleles were amplified in PCR with the same efficiency. We mixed DNA extracts of BO and VOL1 in differing proportions and found a significant linear correlation ($R^2 = 0.9944$; $p < 0.001$) between increasing BO concentration, corresponding to decreasing VOL 1 concentration, and the normalized fluorescence (Fig. 3). DNA extracts of VOL 1 (Pv14 allele size 121) and VOL 2 (Pv14 allele size 125) were also mixed in different proportions and for this alleles couple we obtained similar results of significant correlation ($R^2 = 0.9860$; $P < 0.001$) between the increasing VOL 2 concentration and the corresponding normalized fluorescence (data not shown). The light emission peaks not only provide information on the presence or absence of specific alleles, but their fluorescence intensity is in proportion to the amount of DNA in the original sample. The good correlation found allowed us to use the competitive microsatellite PCR with the SSR marker Pv14 to quantify two genotypes in a co-inoculation experiment. The contribution of each *P. viticola* genotype to infection was expressed as the fluorescence of its allele as a proportion of the total sample fluorescence.

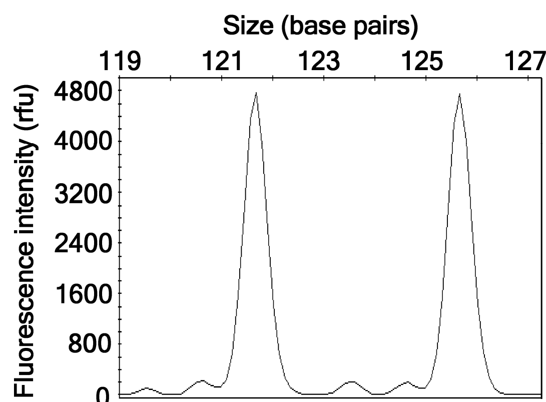

Figure 2. Electropherogram from ABI GeneMapper software illustrating peak sizes (base pairs) and height (fluorescence) of the SSR marker Pv14 on the 1:1 DNA mixture of *Plasmopara viticola* genotypes BO (size 125.67; height 4740) and VOL 1 (size 121.67; height 4760). Both genotypes are homozygous for the SSR marker Pv14

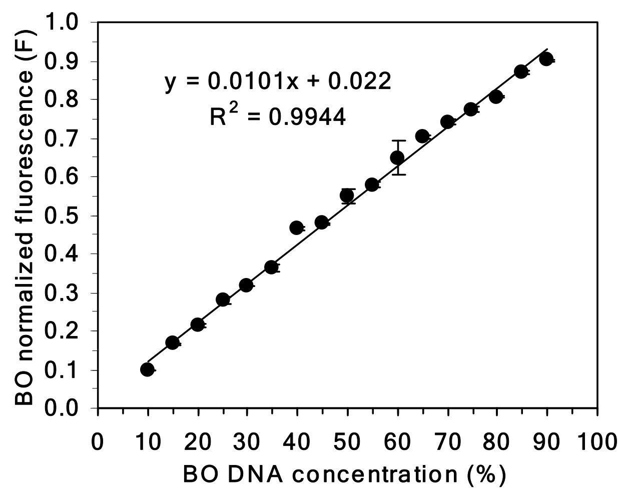

Figure 3. Normalized fluorescence values and linear regression analysis for the SSR marker Pv14 of *Plasmopara viticola* DNA genotype BO (allele size 125) mixed with genotype VOL 1 (allele size 121) in proportions ranging from 10 to 90% at intervals of 5%. Mean values and standard error of three PCR replicates are reported

Co-inoculation dynamics of *Plasmopara viticola* genotypes

Competitive microsatellite PCR gave a precise estimation of the biomass of different *P. viticola* genotypes co-inoculated in the same plant tissue (Fig. 4). BO and VOL 1 genotypes were co-inoculated in five different starting proportions (Fig. 4a) and at each consecutive infection event (from inoculum to the third infection cycle) there was an increasing prevalence of genotype BO over genotype VOL 1. When the initial concentration of BO was greater than 50%, it took two consecutive infection events for BO to predominate over VOL 1. When the initial concentration of BO was 53 and 36%, BO prevailed at the third infection cycle, while at an initial concentration of 11%, BO gradually prevailed over VOL 1 until the last infection cycle, although not completely (89%).

In a second co-inoculation experiment (Fig. 4b), the VOL 1 was co-inoculated with a second isolate deriving from the same vineyard (VOL 2), and an increased prevalence of the genotype VOL 2 over the genotype VOL 1 was obtained at three different starting proportions. In the first two cycles, where VOL 2 concentration was 84%, the initial proportions remained quite stable, however, at the third infection cycle, VOL 2 concentration reach a value higher than 98%. When initial concentration was 53% the VOL 2 concentration remains stable until the first cycle, then in the following cycles its frequency gradually increases to the value of 98%, being practically the only genotype present in the infection. When VOL 2 was present at 31% in the

starting inoculum, gradually its concentration increases reaching the value of 91% at the third infection cycle. Also in this co-inoculation experiment VOL 1 tends to disappear after repetitive infection cycles, permitting to VOL 2 to prevail. Control plants were inoculated with pure BO, VOL 1 and VOL 2 genotypes, resulting in successful infection and production of sporangia at every infection event. Moreover, analysis of the inoculum suspensions and infected leaves confirmed the absence of cross-contamination between genotypes during the experiment (data not shown).

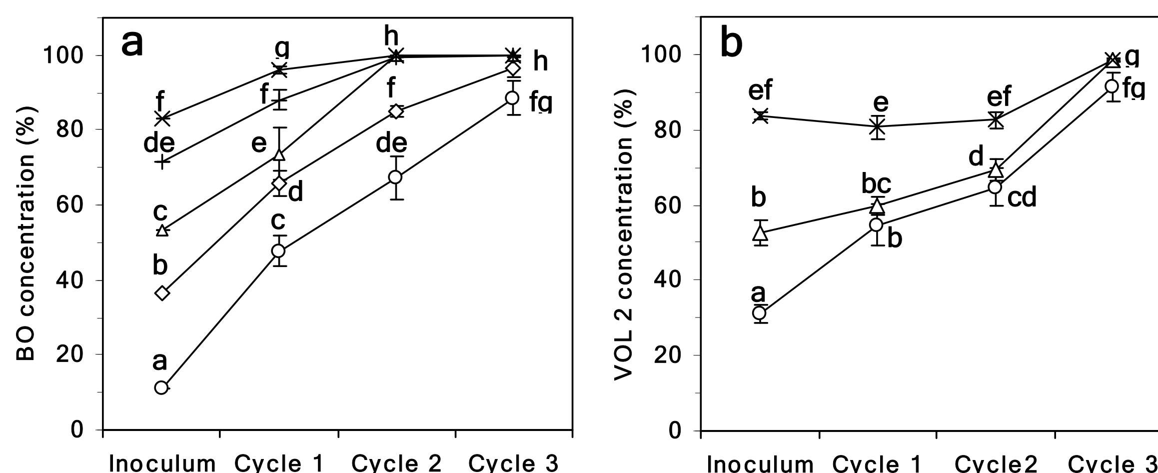

Figure 4. Different proportions of *Plasmopara viticola* genotypes BO (a) and VOL 2 (b) expressed as percentages of total infections (BO + VOL 1 and VOL 1 + VOL 2) inoculated on grapevine leaves followed by three asexual infection cycles. Different starting proportions of the two genotypes were used in the two infection experiments. At each infection cycle, sporangia were harvested separately from each infected leaf and used to inoculate new single leaves. Each point represents mean and standard error of three infected leaves of the same plant. Different letters indicate significant differences according to a Fisher's test

DISCUSSION

In a given *P. viticola* population, particular genotypes dominate while others disappear and never produce secondary lesions (Gobbin et al., 2005). This could be attributed to differences in aggressiveness components, such as sporulation, duration of latency (Corio-Costet et al., 2011; Pariaud et al., 2009) or could be due to external factors such as resistance to applied fungicides or differences in microclimate conditions (Gessler et al., 2011). We initially tested the genotypes collected in different geographical locations (northern, central and southern Italy, and Israel) to ascertain whether different strains had adapted to grow at different temperatures and we

were able to exclude the temperature as a factor affecting the growth (latency) of the isolates. Apart from external factors potentially influencing growth and self-reproduction, other factors such as competition between genotypes during asexual cycles may contribute to the dominance of a given genotype, indicating that additional, as yet identified, processes are playing a role. We therefore studied the dynamics of co-inoculations by *P. viticola* genotypes with similar aggressiveness components in order to assess potential competition during infection. To have a precise estimation of genotypes aggressiveness we referred to the measure of three quantitative traits of pathogen's life cycle such as infection efficiency, latent period and sporulation rate (Pariaud et al., 2009). From these different parameters, a composite fitness index (Corio-Costet et al., 2011) was calculated for each genotype, giving, as result, that the three genotypes used in our co-inoculations experiments were having similar aggressiveness therefore, theoretically, the same chances to successfully produce an infection, grow within the plant tissue and produce sporangia. To quantify the biomasses of the genotypes co-inoculated in the host, we developed a precise method based on competitive microsatellite PCR. Competitive PCR is a precise quantification tool (Zentilin and Giacca 2007) and, given the natural length polymorphism of the SSR marker Pv14, differently-sized microsatellite DNA deriving from different *P. viticola* genotypes can serve as mutual competitors. The method allowed us to differentiate and quantify the biomasses of different *P. viticola* genotypes within the same plant tissue with sufficient resolution to study their variation over time. This quantification method requires careful selection of microsatellite markers as they often generate stutter peaks, which can mask or overlap onto other alleles, and shorter alleles can be preferentially amplified (Daniels et al., 1998). These drawbacks were circumvented in our PCR assay by selecting genotypes presenting similar, but still clearly distinguishable, fragment lengths in homozygosis for the SSR marker Pv14, which were also suitable (sharp peak and low stutter bands) for the quantification method based on competitive PCR. Unlike Naef et al. (2006) and Reininger et al. (2011) we directly quantified the biomasses of the genotypes in the experimental samples without interpolation on the calibration curve. The fluorescence values obtained with the electropherogram were internally normalized and comparison with a standard was not necessary, on the assumption that the sum of the two peaks is equal to 100 and fluorescence of each peak is expressed as a percentage.

When *P. viticola* genotypes were individually inoculated, infections and subsequent sporulation occurred throughout the experiments at comparable rates. However, in both co-inoculation experiments, the two genotypes strongly compete, indicating the existence of other factors for genotype selection. Analysis of three consecutive co-inoculation cycles in two different co-inoculation experiments revealed a decreased frequency of the VOL 1 genotype in the infections, independently of the proportions in the initial inoculum. Whereas the tested genotypes had comparable aggressiveness components and fitness indexes under individual inoculations, VOL 1 had the part of the weaker competitor in the co-inoculation experiments, indicating the additional factors that may play a key role in competition. Although the mechanisms by which different genotypes compete are not well understood, for other oomycetes (i.e. *Phytophthora infestans*) it has been hypothesized that some genotypes could induce production of defense proteins in the host plant earlier than others in a competitive situation (Young et al., 2009). Genotypes that are able to overcome the earlier plant defense reaction would have a competitive advantage, which, coupled with subsequent inhibition of other genotypes, would explain competitive selection (Young et al., 2009). In the pathogenic process caused by *Peronosporaceae*, effector proteins are responsible for modulation of plant cell defense and/or induction of cell death thus creating a favorable environment for infection (Stassen and Van den Ackerveken 2011). *P. viticola* does seem to modulate host cell defenses through apoplastic and cytoplastic effectors secreted during the first stages of infection (Dìez-Navajas et al., 2008) and a first identification of effector genes have been recently described in germinating zoospores (Mestre et al., 2012). The genetic variability of effectors across isolates has been demonstrated in various oomycete species (Halterman et al., 2010) and in *P. infestans* isolates genetic variability at one effector locus has also been correlated with pathogen aggressiveness (Halterman et al., 2010). Thus, as hypothesized for *P. infestans* genotypes (Young et al., 2009), the more competitive *P. viticola* genotypes could have a more complex arsenal of effectors enabling it to suppress or overcome host resistance, while the less competitive genotypes are more vulnerable to the host defense mechanism triggered by the more competitive genotype. The results and interpretations presented here represent a valuable addition to current knowledge of the biology of this grapevine pathogen. Plant-pathogen interactions seem to be more complex than mere colonization of plant tissue, since different *P. viticola* genotypes react differently when they co-exist on a unique substrate. Thus, differences in

the production of effector molecules could contribute to the establishment of dominant genotypes in the field over the course of an epidemic season of downy mildew.

ACKNOLEDGEMENTS

This research was supported by the EnviroChange Project funded by the Autonomous Province of Trento.

REFERENCES

Antonovics, J. and Alexander, H. M. (1989) The concept of fitness in plant-fungal pathogen systems. In: Plant disease epidemiology, Mc-Graw-Hill, 185-214

Corio-Costet, M.-F., Dufour, M.-C., Cigna, J., Abadie, P. and Chen, W.-J. (2011). Diversity and fitness of *Plasmopara viticola* isolates resistant to QoI fungicides. Eur. J. Plant Pathol. 2: 315-329

Daniels, J., Holmans, P., Williams, N., Turic, D., McGuffin, P., Plomin, R. and Owen, M. J. (1998). A simple method for analyzing microsatellite allele image patterns generated from DNA pools and its application to allelic association studies. Am. J. Hum. Genet. 5: 1189-1197

Delmotte, F., Chen, W. J., Richard-Cervera, S., Greif, C., Papura, D., Giresse, X., Mondor-Genson, G. and Corio-Costet, M. F. (2006). Microsatellite DNA markers for *Plasmopara viticola*, the causal agent of downy mildew of grapes. Mol. Ecol. Notes. 2: 379-381

Dìez-Navajas, A. M., Wiedemann-Merdinoglu, S., Greif, C. and Merdinoglu, D. (2008). Nonhost versus host resistance to the grapevine downy mildew, *Plasmopara viticola*, studied at the tissue level. Phytopathology. 7: 776-780

Gessler, C., Pertot, I. and Perazzolli, M. (2011). *Plasmopara viticola* : a review of knowledge on downy mildew of grapevine and effective disease management. Phytopathol. Mediterr. 3-44

Gobbin, D., Bleyer, G., Keil, S., Kassemeyer, H. H. and Gessler, C. (2007). Evidence for sporangial dispersal leading to a single infection event and a sudden high incidence of grapevine downy mildew. Plant Pathol. 5: 843-847

Gobbin, D., Jermini, M., Loskill, B., Pertot, I., Raynal, M. and Gessler, C. (2005). Importance of secondary inoculum of *Plasmopara viticola* to epidemics of grapevine downy mildew. Plant Pathol. 4: 522-534

Gobbin, D., Pertot, I. and Gessler, C. (2003). Identification of microsatellite markers for *Plasmopara viticola* and establishment of high throughput method for SSR analysis. Eur. J. Plant Pathol. 2: 153-164

Gobbin, D., Rumbou, A., Linde, C. C. and Gessler, C. (2006). Population genetic structure of *Plasmopara viticola* after 125 years of colonization in European vineyards. Mol. Plant Pathol. 6: 519-531

Halterman, D. A., Chen, Y., Sopee, J., Berduo-Sandoval, J. and Sànchez-Pérez, A. (2010). Competition between *Phytophthora infestans* Effectors leads to increased aggressiveness on plants Ccontaining broad-spectrum late blight resistance. PLoS ONE. 5: e10536

Laviola, C., Cannizzaro, G., Conigliaro, G. and Burruano, S. (2006). Simple techniques for long-term storage of *Plasmopara viticola*. Phytopathol. Mediterr. 271-275

Matasci, C., Jermini, M., Gobbin, D. and Gessler, C. (2010). Microsatellite based population structure of *Plasmopara viticola* at single vine scale. Eur. J. Plant Pathol. 4: 501-508

Mestre, P., Piron, M.-C. and Merdinoglu, D. (2012). Identification of effector genes from the phytopathogenic Oomycete *Plasmopara viticola* through the analysis of gene expression in germinated zoospores. Fungal Biology. 7: 825-835

Naef, A., Senatore, M. and Défago, G. (2006). A microsatellite based method for quantification of fungi in decomposing plant material elucidates the role of *Fusarium graminearum* DON production in the saprophytic competition with *Trichoderma atroviride* in maize tissue microcosms. FEMS Microbiol. Ecol. 2: 211-220

Pariaud, B., Ravigné, V., Halkett, F., Goyeau, H., Carlier, J. and Lannou, C. (2009). Aggressiveness and its role in the adaptation of plant pathogens. Plant Pathol. 3: 409-424

Perazzolli, M., Roatti, B., Bozza, E. and Pertot, I. (2011). *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defense without costs for grapevine. Biol. Control. 1: 74-82

Reininger, V., Grünig, C. and Sieber, T. (2011). Microsatellite-based quantification method to estimate biomass of *Endophytic Phialocephala* species in strain mixtures. Microb. Ecol. 3: 676-683

Reuveni, M. (2003). Activity of the new fungicide benthiavalicarb against *Plasmopara viticola* and its efficacy in controlling downy mildew in grapevines. Eur. J. Plant Pathol. 3: 243-251

Rumbou, A. and Gessler, C. (2004). Genetic dissection of *Plasmopara viticola* population from a greek vineyard in two consecutive years. Eur. J. Plant Pathol. 4: 379-392

Rumbou, A. and Gessler, C. (2006). Particular structure of *Plasmopara viticola* populations evolved under Greek island conditions. Phytopathology. 5: 501-509

Stassen, J. H. M. and Van den Ackerveken, G. (2011). How do oomycete effectors interfere with plant life? Curr. Opin. Plant Biol. 4: 407-414

Wong, F. P., Burr, H. N. and Wilcox, W. F. (2001). Heterothallism in *Plasmopara viticola*. Plant Pathol. 4: 427-432

Young, G. K., Cooke, L. R., Kirk, W. W., Tumbalam, P., Perez, F. M. and Deahl, K. L. (2009). Influence of competition and host plant resistance on selection in *Phytophthora infestans* populations in Michigan, USA and in Northern Ireland. Plant Pathol. 4: 703-714

Zentilin, L. and Giacca, M. (2007). Competitive PCR for precise nucleic acid quantification. Nat. Protocols. 9: 2092-2104

Chapter 3

Abiotic stresses affect *Trichoderma harzianum* T39-induced resistance to downy mildew in grapevine

Benedetta Roatti, Michele Perazzolli, Cesare Gessler, Ilaria Pertot

Accepted for publication on June 14th (2013) in Phytopathology. DOI:
http://dx.doi.org/10.1094/PHYTO-02-13-0040-R

ABSTRACT

Enhancement of plant defense through application resistance inducers seems a promising alternative to chemical fungicides for controlling crop diseases, but the efficacy can be affected by abiotic factors in the field. Plants respond to abiotic stresses with hormonal signals that may interfere with the mechanisms of induced systemic resistance (ISR) to pathogens. In this study we exposed grapevines to heat and/or drought to investigate the effects of abiotic stresses on grapevine resistance induced by *Trichoderma harzianum* T39 (T39) to downy mildew. Whereas the efficacy of T39-induced resistance was not affected by exposure to heat or drought, it was significantly reduced by combined abiotic stresses. Decrease of leaf water potential and up-regulation of heat-stress markers confirmed that plants reacted to abiotic stresses. Basal expression of defense-related genes and their up-regulation during T39-induced resistance were attenuated by abiotic stresses, in agreement with the reduced efficacy of T39. The evidence reported here suggests that exposure of crops to abiotic stress should be carefully considered to optimize the use of resistance inducers, especially in view of future global climate changes. Expression analysis of ISR markers genes could be helpful to identify when plants are responding to abiotic stresses, in order to optimize treatments with resistance inducers in field.

INTRODUCTION

Grapevines are often exposed to environmental stresses, which may alter their physiological status and affect proper development (Banilas et al., 2012). High temperatures and water deficiency are significant factors limiting plant growth and productivity under field conditions (Ciais et al., 2005). Moreover, global climate change is expected to bring about increases temperatures and reductions in precipitation in several areas of the world (Intergovernmental Panel on Climate Change (IPCC) 2007; Lobell et al., 2011), increasing the risk for heat and drought stresses in plants (Flexas et al., 2009; Liu et al., 2012). Plants have evolved complex systems to defend themselves against abiotic stresses. The cellular processes triggered in response to abiotic stresses take place in all plant organs and are mostly driven by abscisic acid (ABA) signaling (Cramer et al., 2011). During their life cycle, plants are also subjected to biotic stresses, such as pathogens and insect attacks. The signaling molecules involved in these defense responses are mainly salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Pieterse et al., 2012). The protective responses of plants exposed to biotic and abiotic stresses are quite complex, and the signaling pathways interact synergistically and antagonistically with each other (Atkinson and Urwin 2012; Fujita et al., 2006). Greater knowledge of the effect of abiotic stresses and ABA signaling on plant-pathogen interactions was gained in recent times (Adie et al., 2007; Eastburn et al., 2011; Mauch-Mani and Mauch 2005; Robert-Seilaniantz et al., 2011; Ton et al., 2009; Yasuda et al., 2008), and evidences indicated that ABA interferes with plant defense responses to both necrotrophic and biotrophic pathogens (Asselbergh et al., 2008; Atkinson and Urwin 2012; Flors et al., 2008; Robert-Seilaniantz et al., 2011). Therefore, the function of ABA is not limited to the regulation of environmental stresses, but it also tunes many plant processes involving both biotic and abiotic stimuli (Asselbergh et al., 2008). In general, plants respond differently to multiple stresses than to individual stresses (Atkinson and Urwin 2012), indicating that environmental stresses should be taken into account, in order to clearly understand plant defense responses to pathogens.

Downy mildew, caused by the biotrophic oomycete *Plasmopara viticola* (Berck. and Curtis) Berl. and de Toni, is one of the most destructive diseases of grapevine (Gessler et al., 2011). Plants are treated with chemical fungicides throughout the growing season to avoid substantial yield losses (Gessler et al., 2011). In an effort to reduce the use of chemicals, interest

has recently focused on alternative approaches, such as methods based on the enhancement of plant defense (Gessler et al., 2011; Vallad and Goodman 2004). This process relies on the ability of beneficial microorganisms to improve plant self-protection by activating a defense mechanism known as induced systemic resistance (ISR) (Pieterse et al., 2009). ISR can be activated by applying beneficial microorganisms to leaves or roots (Perazzolli et al., 2008; Pieterse et al., 2000) and it is usually regulated by JA- and ET-dependent signaling pathways (Pieterse et al., 2009). ISR is commonly associated with enhanced defense reaction upon pathogen inoculation, also known as priming (Conrath et al., 2006). Application of the ascomycete *Trichoderma harzianum* strain T39 (T39) has been shown to reduce downy mildew symptoms locally and systemically without direct toxic effects on *P. viticola* sporangia, demonstrating that the mechanism of action of T39 to downy mildew was mediated by resistance induction in grapevine (Perazzolli et al., 2008). T39-induced resistance involves JA and ET signaling pathways (Korolev et al., 2008) and does not have negative effects on shoot growth, leaf dimension and chlorophyll content, indicating no energy cost for grapevines (Perazzolli et al., 2011). T39-induced resistance is based on complex reprogramming of the leaf transcriptome and proteome, with direct activation of the microbial recognition machinery and enhanced expression of defense-related processes after downy mildew inoculation (Palmieri et al., 2012; Perazzolli et al., 2012). In particular, T39-induced resistance partially inhibits some disease-related processes and specifically activates defense mechanisms known to be involved in the reaction of resistant genotypes to downy mildew (Perazzolli et al., 2012). Moreover, histological analysis indicated that T39 primes grapevine defenses for enhanced accumulation of callose in stomata guard cells and production of reactive oxygen species (ROS) in leaf tissues upon *P. viticola* inoculation (Palmieri et al., 2012).

Activation of ISR through application of beneficial microorganisms appears to be a promising method for the biological control of crop diseases (Vallad and Goodman 2004). However, disease control mediated by ISR is often inconsistent and only partially effective under field conditions, and employment of this biocontrol tool in agriculture is far from widespread (Gessler et al., 2011; Walters 2009; Walters et al., 2013). ISR is a plant response regulated by a complex crosstalk of hormonal signaling (Pieterse et al., 2009) and it is likely to be affected by environmental conditions and exposure of plants to abiotic stresses (Walters et al., 2005; Walters 2009; Walters et al., 2013). Greater knowledge of the ISR efficacy in plants exposed to various

abiotic conditions is particularly important to further implement the use of resistance inducers in plant protection strategies in field conditions (Walters et al., 2005; Walters 2009; Walters et al., 2013). The aim of this study was to investigate the effect of short exposure of grapevine plants to heat and drought stress on the level of resistance induced by T39 treatment against downy mildew. Our physiological and molecular analysis showed that T39-induced resistance was negatively affected by exposure of plants to combined abiotic stresses, suggesting that environmental conditions should be carefully considered to further optimize the use of resistance inducers in vineyards, especially in view of future climate changes.

MATERIALS AND METHODS

Exposure of grapevines to heat and drought stress

Two-year-old plants of the susceptible grapevine cultivar *V. vinifera* cv. Pinot Noir grafted onto Kober 5BB were planted in individual 2.5 L pots containing a mixture of peat and pumice (3:1). Plants were grown in the greenhouse at 25 ± 1 °C with a relative humidity (RH) of 60 ± 10% for two months. Plants were then incubated for 14 days under the following temperature and irrigation conditions: unstressed (NS: irrigation at field capacity every two days at 25 ± 1 °C); drought stress (no irrigation at 25 ± 1 °C); heat stress (daily irrigation at field capacity at 35 ± 1 °C); heat + drought stress (HDS, daily irrigation with 50 mL of water at 35 ± 1 °C). HDS plants were irrigated with this small amount of water to prevent wilting. RH was 60 ± 10% at 25 °C and 50 ± 10% at 35 °C.

Analysis of soil moisture, leaf water potential and leaf chlorophyll content

Soil moisture was measured with a Time Domain Reflectometry (TDR) portable meter (Field Scout TDR 200 Soil Moisture Probe, Spectrum Technologies, USA) as volumetric water content. TDR exploits the electrical conductivity of the soil solution (which varies as a function of soil moisture) to calculate the volumetric water content of the soil, expressed as a percentage. Volumetric water content of each pot was measured daily at the same time using 15 cm rods to monitor the irrigation scheduling.

Mid-morning (four hours after light onset) total leaf water potential (Ψ_{leaf}) was measured using a Scholander-type pressure chamber (Soil Moisture Equipment Corp, Santa Barbara, CA,

USA) accordingly to Lovisolo et al. (Lovisolo et al., 2002). Measurements were taken from 1000 to 1130 HR under a light intensity (photosynthetic active radiation) of 30 μmol m^{-2} s^{-1}, at the beginning (day 1) and at the end (day 14) of the stressing period. Four plants per growth condition were randomly selected. In each plant, Ψ_{leaf} was measured in three mature primary leaves detached from the 4th, 5th and 6th nodes starting from the apical meristem. Each leaf was put into a plastic bag prior to excision and assessed within 30 seconds (Poni et al., 2009). Mean Ψ_{leaf} was calculated for each plant and mean Ψ_{leaf} was calculated for each growth condition.

Quantitative assessment of the early stages of plant stress was evaluated by estimating chlorophyll content (Steele et al., 2008). A non destructive SPAD meter (SPAD-502, Minolta Camera Co., Osaka, Japan) was used, and leaf transmittance was measured four hours after light onset on three leaves of each plant (from the 4th, 5th and 6th nodes starting from the apical meristem), at the beginning (day 1) and on day 11 of the stressing period. Leaf chlorophyll content was then obtained by converting SPAD measures in mg of chlorophyll per m^2 of leaf area (mg m^{-2}), according to the relationship optimized for grapevine leaves (Steele et al., 2008).

Resistance induction and control treatments

T. harzianum T39 was grown for two weeks at 25 °C on 100 g of twice sterilized rice grains (Longa et al., 2009). A conidia suspension was obtained by washing the colonised rice grains in distilled water. The rice grains were filtered out with a fine net and the suspension was adjusted to 1×10^7 conidia mL^{-1} by counting with a haemocytometer under a light microscope. Plants were sprayed with the T39 conidia suspension to induce resistance against *P. viticola* (Perazzolli et al., 2011), and control plants were treated with distilled water (control). Treatments started on day 11 of the experiment and were repeated daily for three consecutive days (one, two and three days before *P. viticola* inoculation) in order to induce a high level of phenotypic resistance response (Perazzolli et al., 2008; Perazzolli et al., 2011). Treatments were applied with a compressed air hand sprayer to the abaxial and adaxial surfaces of all leaves; 20-30 mL were applied to each plant depending on the number of leaves. Nine replicates (plants) per treatment per growth condition were analyzed in a randomized complete block design and the experiment was carried out twice.

Pathogen inoculation, assessment of disease severity and efficacy of T39-induced resistance

A *P. viticola* isolate was collected from an untreated vineyard in Trentino (northern Italy) and maintained by subsequent inoculations on *V. vinifera* Pinot Noir plants under greenhouse conditions. Fresh sporangia were obtained by placing plants with oil spot symptoms in the dark overnight at 99-100% RH and 25 ± 1 °C. Sporangia were then collected by washing the leaves bearing sporulating lesions with cold (5 °C) distilled water. The concentration of the inoculum suspension was adjusted to 1×10^5 sporangia ml^{-1} by counting with a haemocytometer under a light microscope.

The day after the last treatment, abaxial leaf surfaces were inoculated with *P. viticola* using a compressed air hand sprayer. Approximately 20-30 mL of inoculum suspension were sprayed onto each plant, depending on the number of leaves. The time point at which the plants were inoculated coincides with the end of the stressing period. Inoculated plants were incubated overnight in the dark at 25 ± 1 °C and 99-100% RH (inoculum conditions), and then maintained under controlled greenhouse conditions at 25 ± 1 °C and RH 60 ± 10%. Six replicates (plants) per treatment per growth condition were inoculated in a randomized complete block design, while the remaining three plants were incubated overnight in the inoculum conditions without being inoculated.

Six days after pathogen inoculation, plants were incubated overnight in the dark at 25 ± 1 °C with 99-100% RH to promote downy mildew sporulation. Severity of downy mildew disease was assessed visually on all leaves of each plant, according to the standard guidelines of the European and Mediterranean Plant Protection Organisation (European and Mediterranean Plant Protection Organization (EPPO) 2001); http://pp1.eppo.int/list.php). For each leaf, disease severity was expressed as the proportion (percentage from 0 to 100%, with intervals of 5%) of abaxial leaf area covered by white sporulation of *P. viticola* in relation to the total leaf area, and a mean value was calculated for each plant (Corio-Costet et al., 2011; Malacarne et al., 2011). Induced resistance was evaluated by analyzing the reduction of disease severity on leaves previously treated with the resistance inducer, and the efficacy of the treatment was calculated for each growth condition according to the following equation: efficacy = (severity of control plants - severity of T39-treated plants) / severity of control plants × 100. The experiment was carried out twice.

Leaf sample collection and gene expression analysis.

Leaf samples from T39-treated and control plants under the NS and HDS conditions were collected immediately before *P. viticola* inoculation (un-inoculated samples at 0 h), which corresponded to the end of the stressing period. Leaf samples were also collected at 24 h after *P. viticola* inoculation from inoculated plants (inoculated samples at 24 h) and from un-inoculated plants maintained under the inoculum conditions (un-inoculated samples at 24 h). Three plants (replicates) per time point were sampled for each treatment per growth condition. Each sample comprised three half-leaves from the same plant, and only leaves of the 4th, 5th and 6th nodes starting from the apical meristem were collected, pooled and immediately frozen in liquid nitrogen and stored at -80 °C.

Total RNA was extracted from the frozen samples using the Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) and quantified by NanoDrop 8000 (Thermo Fisher Scientific, Wilmington, USA). RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA), and the first strand cDNA was synthesised from 1.0 µg of total RNA using Superscript III (Invitrogen) and oligo-dT primer. Quantitative real-time PCR reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and specific primers (Table 1) using the Light Cycler 480 (Roche Diagnostics, Mannheim, Germany). The PCR conditions were: 50 °C for 2 min and 95 °C for 2 min as initial steps, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample was examined in three technical replicates and dissociation curves were analyzed to verify the specificity of each amplification reaction. Ct values were extracted with the Light Cycler 480 SV1.5.0 software (Roche) using the second derivative calculation and reaction efficiency was calculated with the LinRegPCR 11.1 software (Ruijter et al., 2009). The relative expression of each gene was obtained according to the Pfaffl equation (Pfaffl 2001) using *Actin* as reference gene with constitutive expression for normalization (Perazzolli et al., 2012; Polesani et al., 2010). Relative expression values were calculated using NS un-inoculated control plants at 0 h as the calibrator for gene expression analysis at the end of the stressing period. For gene expression analysis during T39-induced resistance, relative expression values were calculated using NS un-inoculated control plants at 24 h as the calibrator. The priming effect was calculated as the ratio between the expression level in inoculated (24 h post-inoculation) leaves of T39-treated plants and the expression level in inoculated control plants, using a threshold of 1.5-fold to identify augmented expressions (Verhagen et al., 2004). For each

sample, mean expression values and standard errors were calculated on three replicates and two independent experiments were analyzed.

Leaf sample collection and gene expression analysis

Leaf samples from T39-treated and control plants under the NS and HDS conditions were collected immediately before *P. viticola* inoculation (un-inoculated samples at 0 h), which corresponded to the end of the stressing period. Leaf samples were also collected at 24 h after *P. viticola* inoculation from inoculated plants (inoculated samples at 24 h) and from un-inoculated plants maintained under the inoculum conditions (un-inoculated samples at 24 h). Three plants (replicates) per time point were sampled for each treatment per growth condition. Each sample comprised three half-leaves from the same plant, and only leaves of the 4th, 5th and 6th nodes starting from the apical meristem were collected and pooled then immediately frozen in liquid nitrogen and stored at -80 °C.

Total RNA was extracted from the frozen samples using the Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) and quantified by NanoDrop 8000 (Thermo Fisher Scientific, Wilmington, USA). RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA), and the first strand cDNA was synthesised from 1.0 µg of total RNA using Superscript III (Invitrogen) and oligo-dT primer. Quantitative real-time PCR reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and specific primers (Table 1) using the Light Cycler 480 (Roche Diagnostics, Mannheim, Germany). The PCR conditions were: 50 °C for 2 min and 95 °C for 2 min as initial steps, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample was examined in three technical replicates and dissociation curves were analyzed to verify the specificity of each amplification reaction. Ct values were extracted with the Light Cycler 480 SV1.5.0 software (Roche) using the second derivative calculation and reaction efficiency was calculated with the LinRegPCR 11.1 software (Ruijter et al., 2009). The relative expression of each gene was obtained according to the Pfaffl equation (Pfaffl 2001) using *Actin* as reference gene with constitutive expression for normalisation (Perazzolli et al., 2012; Polesani et al., 2010). Relative expression values were calculated using NS un-inoculated control plants at 0 h as the calibrator for gene expression analysis at the end of the stressing period. For gene expression analysis during T39-induced resistance, relative expression values were calculated using NS un-inoculated control plants at 24 h as the calibrator. The priming

effect was calculated as the ratio between the expression level in inoculated leaves of T39-treated plants and the expression level in inoculated control plants (24 h post-inoculation), using a threshold of 1.5-fold to identify augmented expressions (Verhagen et al., 2004). For each sample, mean expression values and standard errors were calculated on three replicates and two independent experiments were analyzed.

Primer design for heat stress-related genes

Published sequences of three heat shock proteins (HSP; Table 1) were aligned against the Pinot Noir genome (Velasco et al., 2007) Release 3 (http://genomics.research.iasma.it): heat shock-induced gene 4 (*HSG4*; (Kobayashi et al., 2010), heat shock-induced gene 19 (*HSG19*; (Kobayashi et al., 2010) and the gene encoding the heat shock protein 90 (*HSP90.1a*; (Banilas et al., 2012). Sequences were aligned using ClustalW2 (www.ebi.ac.uk) and primer pairs were designed on the Pinot Noir sequences using the Primer 3 software v. 4.0 (http://primer3.sourceforge.net) and analyzed with the Oligo Analyzer v. 1.0.2 (www.bio.net/bionet/mm/bio-soft/2001-September/023431.html).

Statistical analysis

Two independent experiments were carried out and variance of the data was analyzed using the Statistica 9 software (StatSoft, Tulsa, OK). An F-test was used to demonstrate non-significant treatment–experiment interactions ($P > 0.05$) and data from the repeated experiments were pooled. Values of Ψ_{leaf}, and T39 efficacy were Log_{10} transformed, and fold change values of gene expression analysis were transformed according to the equation $Y = Log_{10}(1 + x)$ (Casagrande et al., 2011). A box and whisker plot was used to detect outliers. Normal distribution was evaluated with the *K-S* test ($P > 0.05$). Data were validated for homogeneity of variance (Leven's test, $P > 0.05$) and analysis of variance (ANOVA) was carried out with a Fisher's test to detect significant differences ($P < 0.05$).

Table 1. Primer sequences for quantitative real time RT-PCR (RT-qPCR) expression analysis of grapevine genes

Name	Abbreviation	Accession number[a]	Pinot Noir gene[b]	RT-qPCR primers		Reference
Pathogenesis-related protein 2	*PR-2*	AJ277900	twinscan.VV78X005385.7_1	*PR-2_For* *PR-2_Rev*	GTTATTTCAGAGAGTGGTTGGC AACATGGCAAACACGTAAGTCT	(Perazzolli et al., 2010; Perazzolli et al., 2011)
Lipooxygenase 9	*LOX-9*	AY159556	twinscan.VV78X044916.31_1	*LOX-9_For* *LOX-9_Rev*	CCCTTCTTGGCATCTCCCTTA TGTTGTGTCCAGGGTCCATTC	(Perazzolli et al., 2011; Trouvelot et al., 2008)
Trichoderma-induced osmotin	*OSM-1*	XM_002282928	glimmer.VV78X132476.3_2	*OSM-1_For* *OSM-1_Rev*	CGCTGCGCTAAAGACTACC AAAAACCTTGAGTAATCTGTAGCA	(Perazzolli et al., 2012)
Pathogenesis-related protein 4	*PR-4*	CF74510	glimmer.VV78X053121.6_1	*PR-4_For* *PR-4_Rev*	CAGGCAACGGTGAGAATAGT ACCACAGTCCACAAACTCGTA	(Perazzolli et al., 2010; Perazzolli et al., 2011)
Acidic endochitinase 3	*CHIT3*	XM_003634041	glimmer.VV78X036668.3_2	*CHIT3_For* *CHIT3_Rev*	GTCCATTCCCAGATAAGTTCCT CAGAAGGTTATTGGTGTTGCC	(Perazzolli et al., 2012)
Ethylene-responsive transcription factor ERF003	*ERF*	XM_002285337	glimmer.VV78X034923.15_2	*ERF_For* *ERF_Rev*	ACCAAACAATCCCTGCATGA GGCTGCTGCACTGTCACC	(Perazzolli et al., 2012)
Heat shock induced gene 4	*HSG4*	GU169699	glimmer.VV78X138978.6_1	*HSG4_For* *HSG4_Rev*	GAATGAAAGTGGCACCGAGT CCTCAGCCTTGGGAACAGTA	(Kobayashi et al., 2010)
Heat shock induced gene 19	*HSG19*	GU169701	glimmer.VV78X000784.5_3	*HSG19_For* *HSG19_Rev*	TATGAGGAAGTTTGCGTTGC TCAATGGTCTTGGGCTTCTT	(Kobayashi et al., 2010)
Heat shock protein 90	*VvHSP90.1a*	XP_002274022	glimmer.VV78X151268.17_1	*HSP90_For* *HSP90_Rev*	CCTCCTGACTTCTGGGTTCA ATGTCAGCATCCTCCTCACC	(Banilas et al., 2012)
Actin	*Act*	TC81781	glimmer.VV78X114914.6_2	*Act_For* *Act_Rev*	ATTCCTCACCATCATCAGCA GACCCCCTCCTACTAAAACT	(Polesani et al., 2010)

[a]Accession numbers refer to the National Center for Biotechnology Information (NCBI) Gene Bank (www.ncbi.nlm.nih.gov).

[b]The corresponding Pinot Noir genes used for primer design were identified by a nucleotide BLAST search against the *Vitis vinifera* Pinot Noir predicted genes (Velasco et al., 2007) of Release 3 (http://genomics.research.iasma.it/).

RESULTS

Soil moisture, leaf water potential and leaf chlorophyll content during the stressing period.

To monitor the effects of irrigation scheduling, soil water content was measured during abiotic stress exposure. The mean soil water content of NS plants and heat-stressed plants was about 45% during the stressing period (Fig. 1A). The soil water content of drought-stressed plants was decreasing to 11% by the end of the stressing period, and that of HDS plants oscillated between 20 and 30%, probably due to the small amount of water given to avoid wilting.

At the end of the stressing period (day 14), Ψ_{leaf} in drought-stressed plants and in HDS plants was significantly lower than in NS plants (Fig. 1B). Values of Ψ_{leaf} indicated the adaptation of grapevines to water stress (Lovisolo et al., 2002; Lovisolo et al., 2008; Perrone et al., 2012) at the end of the drought-stress and HDS condition, and not at the end of heat-stress exposure.

No signs of wilting or of reduced chlorophyll content were visible in plants in any of the stressing conditions: chlorophyll content did not differ significantly between the four growth conditions at day 1 (279 ± 2 mg m^{-2}) and at day 11 (301 ± 4 mg m^{-2}).

Severity of downy mildew and efficacy of T39-induced resistance in grapevines exposed to abiotic stresses.

Values of downy mildew severity and T39 efficacy revealed non-significant treatment–experiment interactions (F-test, $P > 0.05$), and the results of two independent experiments were pooled. Downy mildew severity of control plants treated with distilled water was comparable in NS and abiotic stressed plants (Supplemental Table 1), indicating that the increase of temperature and/or reduction of plant irrigation did not affect pathogen infection and symptoms development. As previously reported (Perazzolli et al., 2008; Perazzolli et al., 2011), foliar application of T39 significantly reduced downy mildew severity compared with control plants under NS condition. T39 treatment reduced disease severity also in plants exposed to single abiotic stress (drought or heat), but not in HDS plants (Supplemental Table 1). In particular, the efficacy of T39-induced resistance in NS plants was 53 ± 7 % (Fig. 2): the extent of downy mildew sporulation on NS T39-treated plants was about half that on NS control plants.

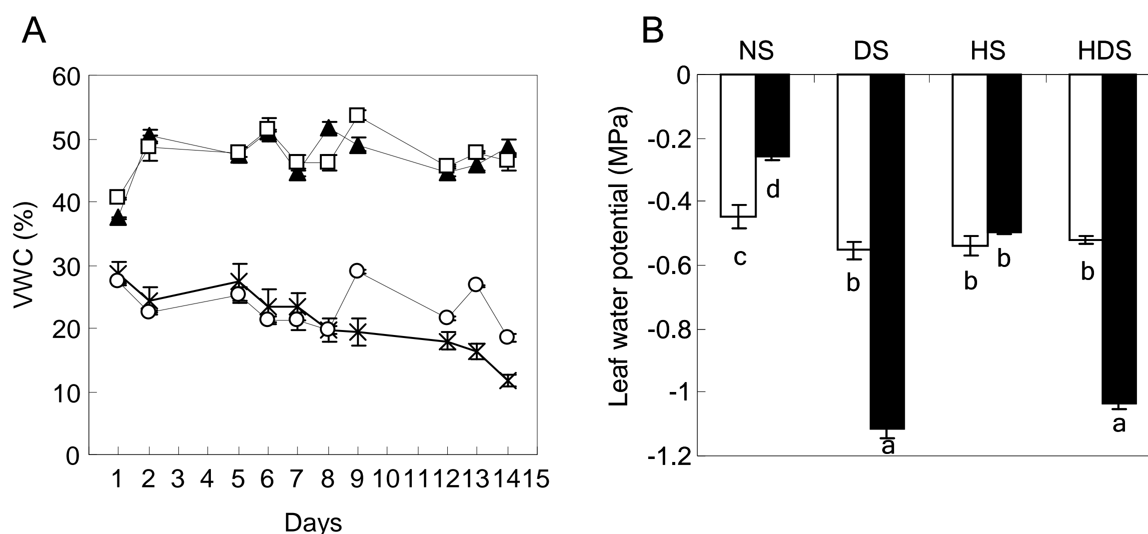

Figure 1. Soil water content (% of volume, **A**) measured in pots of Pinot Noir grapevine plants subjected to unstressed conditions (▲), drought stress (×), heat stress (□), heat + drought stress (○) for 14 days. Each point represents the mean and standard error of 36 replicates (plants) pooled from two independent repetitions of the experiment. Leaf water potential (Ψ_{leaf}; **B**) was measured in grapevine plants subjected to unstressed conditions (NS), drought stress (DS), heat stress (HS), heat + drought stress (HDS) for 14 days. Three leaves per plant were analyzed at the beginning (white, day 1) and at the end (black, day 14) of the stressing period. F-test revealed non-significant treatment-experiment interactions ($P > 0.05$) and data from two repetitions of the experiment were pooled. Values are means of eight plants pooled from two independent repetitions of the experiment. Different letters indicate significant differences according to a Fisher's test ($P < 0.05$) on Log_{10} transformed values.

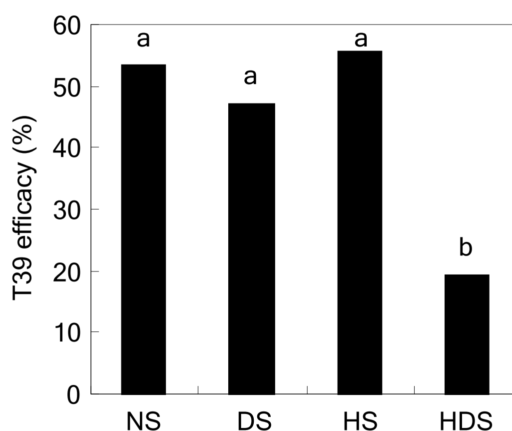

Figure 2. Efficacy of *Trichoderma harzianum* T39 (T39) against downy mildew in grapevine plants exposed to unstressed (NS), drought-stressed (DS), heat-stressed (HS), and heat + drought stressed (HDS) conditions. Downy mildew severity was assessed on all leaves of each plant as percentage of abaxial leaf area covered by sporulation of downy mildew (Supplemental Table 1). Efficacy of T39 was calculated for each growth condition accordingly to the equation: efficacy = (severity of control plants - severity of T39-treated plants) / severity of control plants × 100. F-test revealed non-significant treatment-experiment interactions ($P > 0.05$) and data from two repetitions of the experiment were pooled. Each bar represents the mean of 12 replicates (plants) pooled from two independent repetitions of the experiment. Different letters indicate significant differences among treatments and growth conditions, according to Fisher's test ($P < 0.05$).

The efficacy of T39-induced resistance in drought-stressed and heat-stressed plants was comparable to NS plants, indicating that the increase in environmental temperature or reduction in plant irrigation did not affect interaction with the beneficial microorganisms and the level of induced resistance in grapevine. Interestingly, the efficacy of T39-induced resistance to downy mildew was significantly lower in HDS plants (19 ± 1 % of T39 efficacy) than in NS plants, indicating the negative effect of exposure to combined abiotic stresses.

Modulation of heat-stress markers and defense-related genes in grapevines exposed to abiotic stresses.

Gene expression analysis was carried out on leaves collected on the last day of the stressing period, immediately before *P. viticola* inoculation (Table 2 and Fig. 3). NS and HDS plants of both control and T39-treated plants were analyzed. The gene expression levels of *HSG4*, *HSG19* and *HSP90.1a* were induced in HDS control and HDS T39-treated plants, while they remained at constitutive levels in NS control and NS T39-treated plants (Table 2), demonstrating that 35 °C caused heat stress for plants.

The defense-related genes were modulated in NS and HDS plants before pathogen inoculation (Fig. 3). A 7-fold repression of the gene encoding the pathogenesis-related protein 2 (*PR-2*) was observed in HDS control plants compared with NS control plants (Fig. 3A). Moreover, the level of *PR-2* expression was 1.9-fold lower in HDS T39-treated compared with NS control plants.

A significant repression (2.9-fold) of lipoxygenase 9 (*LOX-9*) was observed in HDS control plants compared with NS control plants (Fig. 3B). *LOX-9* is a marker of JA signaling (Hamiduzzaman et al., 2005; Perazzolli et al., 2011) and its expression level in HDS T39-treated plants was similar to NS control plants. However, *LOX-9* was not induced by T39 treatment neither in HDS nor NS plants.

The *Trichoderma*-induced osmotin 1 (*OSM-1*) was repressed (2.4-fold) in HDS control plants compared with NS control plants (Fig. 3C). Grapevine *OSM-1* is a marker of T39-induced resistance (Perazzolli et al., 2012) and it was induced by T39 treatment in both NS (3.8-fold) and HDS (2.0-fold) plants compared with NS control plants. Interestingly, the expression level of *OSM-1* in T39-treated plants was significantly lower in the HDS than in the NS plants.

Table 2. Relative expression levels of heat shock marker genes in unstressed (NS) and heat + drought-stressed grapevine plants (HDS) treated with distilled water (control) or with *Trichoderma harzianum* T39 (T39).

Gene name	Abbreviation	NS control		HDS control		NS T39-treated		HDS T39-treated	
Heat shock-induced gene 4	*HSG4*	1.2 ± 0.7	c	80.4 ± 1.5	b	0.7 ± 0.1	c	63.8 ± 2.7	a
Heat shock-induced gene 19	*HSG19*	1.1 ± 0.5	b	25.7 ± 1.4	a	0.7 ± 0.1	b	21.0 ± 3.5	a
Heat shock protein 90.1a	*HSP90.1a*	2.3 ± 1.1	b	520.7 ± 25.4	a	2.5 ± 0.5	b	433.9 ± 106.1	a

[a]Relative expression levels (fold changes) were calculated using *Actin* as constitutive gene for normalization and data were calibrated on un-inoculated NS control plants at 0 h. Mean levels of relative expression and standard errors were calculated from six replicates (plants) per treatment pooled from two independent repetitions of the experiment. For each gene, different letters indicate significant differences according to a Fisher's test ($P < 0.05$) (Casagrande et al., 2011).

No significant modulation of the gene encoding the pathogenesis-related protein 4 (*PR-4*) was observed in HDS control plants compared with NS control plants (Fig. 3D). *PR-4* is a marker of JA signaling known to be involved in T39-induced resistance (Perazzolli et al., 2011) and it was significantly induced by T39 treatment in both NS and HDS plants. Likewise, no changes in acid endochitinase 3 (*CHIT3*) gene expression were observed in HDS control plants compared with NS control plants (Fig. 3E). The *CHIT3* gene is induced by T39 in grapevine (Perazzolli et al., 2012) and in our experiments it was induced by T39 treatment in both NS and HDS plants. The level of *CHIT3* expression did not vary between HDS plants and NS plants, and it was 2-fold higher in T39-treated than in control samples. The ethylene-responsive transcription factor (*ERF*) was not modulated in T39-treated and control plants neither in NS nor in HDS conditions (Fig. 3F), in agreement with the finding that this gene is modulated only after pathogen inoculation in T39-treated grapevines (Perazzolli et al., 2012).

Modulation of defense-related genes during *Trichoderma harzianum* T39-induced resistance under stress conditions.

Expression of the above-mentioned defense-related genes was analyzed in leaves collected at 24 h after *P. viticola* inoculation (Fig. 4). As the plants were kept under the same environmental conditions during inoculation, heat and drought stress had ceased in the leaves sampled 24 h after *P. viticola* inoculation. *PR-2* was significantly induced by *P. viticola* in NS control plants, but not in HDS control plants (Fig. 4A). The highest level of *PR-2* expression was observed in inoculated NS T39-treated plants with a priming effect of 1.9-fold compared with

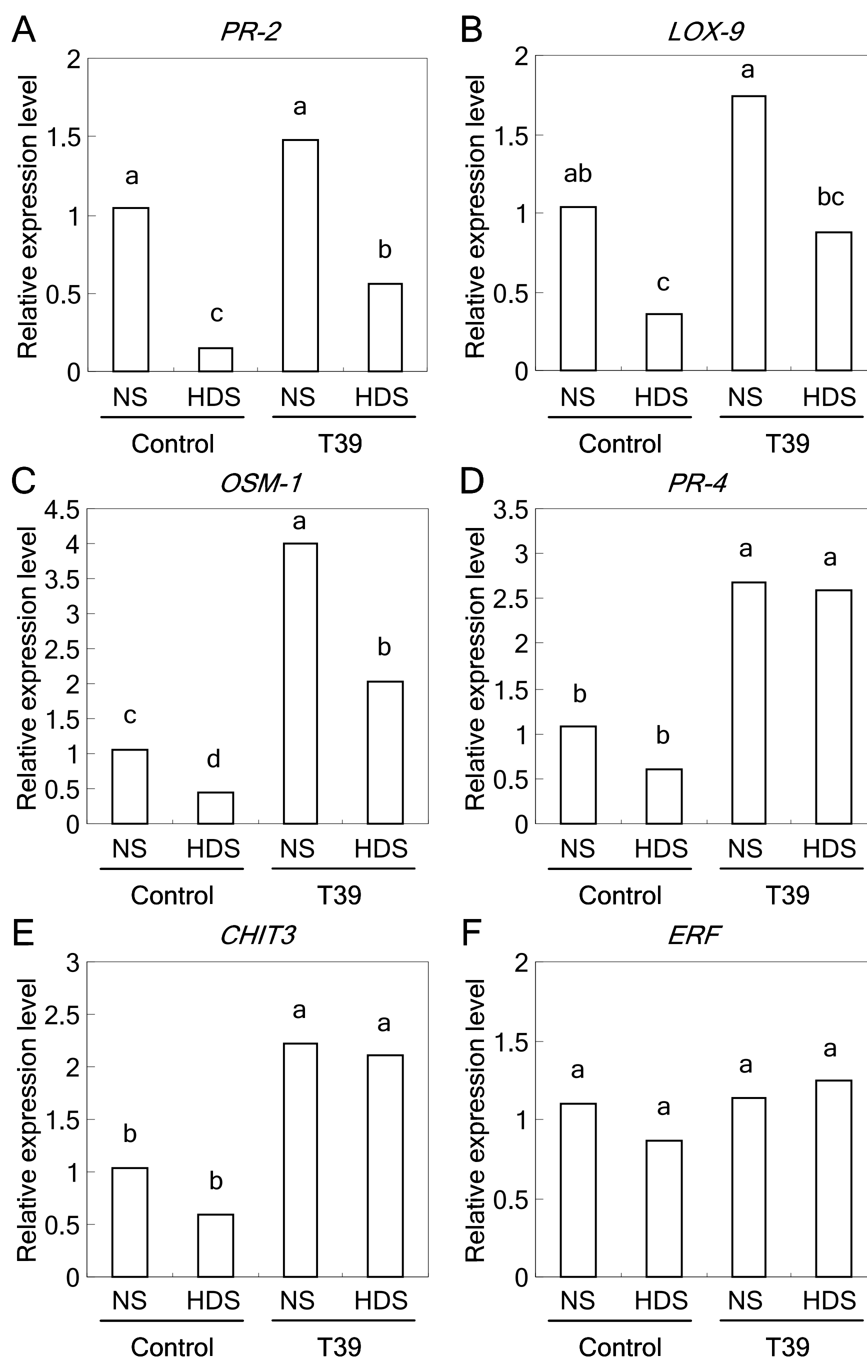

Figure 3. Gene expression analysis of genes encoding pathogenesis-related protein 2 (*PR-2*; **A**), lipoxygenease 9 (*LOX-9*; **B**), *Trichoderma*-induced osmotin 1 (*OSM-1*; **C**), pathogenesis-related protein 4 (*PR-4*; **D**), acid endochitinase 3 (*CHIT3*; **E**), and ethylene response factor (*ERF*: **F**) in Pinot Noir grapevine plants. Analysis was performed on unstressed (NS) and heat + drought-stressed (HDS) grapevine plants treated with distilled water (Control) or with *Trichoderma harzianum* T39 (T39). Relative expression levels (fold changes) were calculated using *Actin* as constitutive gene for normalization and data were calibrated on un-inoculated NS control plants at 0 h. F-test revealed non-significant treatment-experiment interactions ($P > 0.05$) and data from two repetitions of the experiment were pooled. Mean levels of relative expression were calculated from six replicates (plants) per treatment pooled from two independent repetitions of the experiment. For each gene, different letters indicate significant differences according to Fisher's test ($P < 0.05$) on fold change values transformed according to the equation $Y = Log_{10} (1 + x)$ (Casagrande et al., 2011).

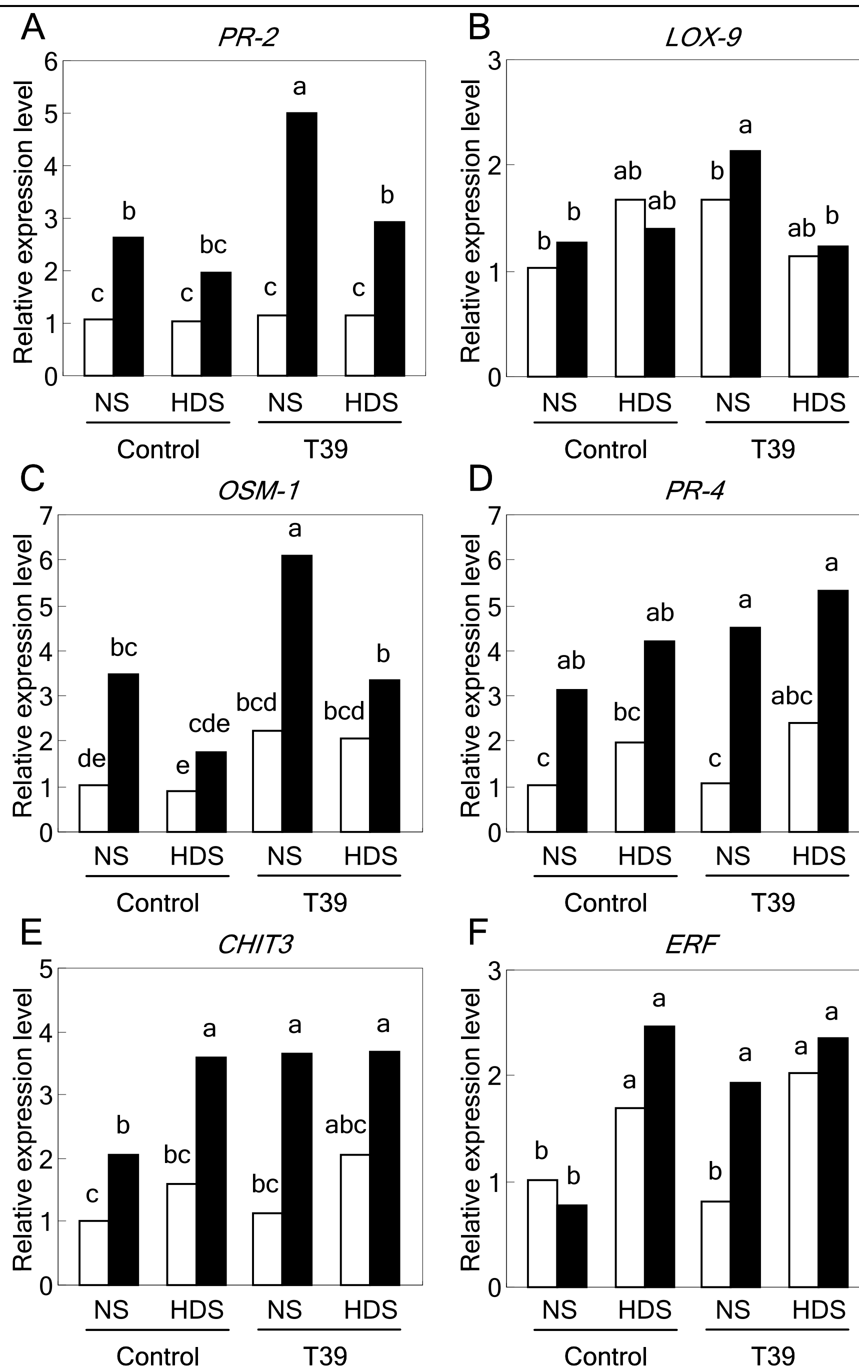

Figure 4. Gene expression analysis of genes encoding pathogenesis-related protein 2 (*PR-2*; **A**), lipoxygenease 9 (*LOX-9*; **B**), *Trichoderma*-induced osmotin 1 (*OSM-1*; **C**), pathogenesis-related protein (*PR-4*; **D**), acid endochitinase 3 (*CHIT3*; **E**) and ethylene response factor (*ERF*; **F**) in Pinot Noir grapevine plants either un-inoculated (white) or inoculated with *Plasmopara viticola* (black) at 24 h. Analysis was performed on unstressed (NS) and heat + drought-stressed grapevine plants (HDS) treated with distilled water (Control) or with *Trichoderma harzianum* T39 (T39). Relative expression levels (fold changes) were calculated using *Actin* as constitutive gene for normalization and data were calibrated on un-inoculated NS control plants at 24 h. F-test revealed non-significant treatment-experiment interactions ($P > 0.05$) and data from two repetitions of the experiment were pooled. Mean levels of relative expression were calculated from six replicates (plants) per treatment pooled from two independent repetitions of the experiment. For each gene, different letters indicate significant differences according to Fisher's test ($P < 0.05$) on fold change values transformed according to the equation $Y = Log_{10} (1 + x)$ (Casagrande et al., 2011).

inoculated NS control plants. In T39-treated plants, the expression level of *PR-2* after inoculation was lower in the HDS than in the NS plants.

LOX-9 is usually induced by *P. viticola* inoculation (Hamiduzzaman et al., 2005; Perazzolli et al., 2011), and in our experiments it was significantly induced only in NS T39-treated plants after inoculation (Fig. 4B). *LOX-9* was not significantly affected by the abiotic stresses in un-inoculated plants, and its expression after inoculation was lower in HDS T39-treated that in NS T39-treated plants.

OSM-1 was not modulated in un-inoculated control plants and induction caused by T39 was not significant, neither in NS nor in HDS plants at 24 h (Fig. 4C). The highest level of *OSM-1* expression was observed in NS T39-treated plants after *P. viticola* inoculation, with a significant priming effect of 1.8-fold compared with inoculated NS control plants. In *P. viticola*-inoculated samples, the expression level of *OSM-1* was lower in HDS T39-treated than in NS T39-treated plants.

PR-4 was not significantly modulated in un-inoculated plants at 24 h and it was induced by *P. viticola* inoculation with similar expression levels in all tested conditions (Fig. 4D). *CHIT3* was not significantly modulated in un-inoculated plants in any of the tested conditions, and it was induced by *P. viticola* in NS control plants (Fig. 4E). In *P. viticola*-inoculated samples, the expression level of *CHIT3* was higher in NS T39-treated than in NS control plants, with a significant priming effect of 1.8-fold. Under HDS conditions, *CHIT3* was induced by *P. viticola* in both T39-treated and control plants, but no significant priming effect was observed compared to HDS inoculated control plants. *ERF* was induced by *P. viticola* inoculation in T39-treated but not in control plants under NS conditions (Fig. 4F), in agreement with Perazzolli et al. (Perazzolli et al., 2012). Moreover, induction of *ERF* was also observed in both inoculated and un-inoculated HDS plants.

DISCUSSION

Trichoderma species have been recognized as biocontrol agents for several pathogens trough different mechanisms of action (Shoresh et al., 2010). The T39 strain reduces downy mildew severity in grapevine by inducing systemic resistance, without any direct toxic effect on *P. viticola* sporangia (Perazzolli et al., 2008). T39-induced resistance is mediated by JA and ET

signaling pathways (Korolev et al., 2008) and does not entail energy costs for grapevine plants, suggesting minimal deleterious effects on grape production and quality (Perazzolli et al., 2011). The mechanisms of T39-induced resistance are based on a complex reprogramming of the leaf transcriptome (Perazzolli et al., 2012) and proteome (Palmieri et al., 2012), with early activation of plant defense processes (e.g. accumulation of callose and ROS) after downy mildew inoculation (Palmieri et al., 2012). Resistance induced by beneficial microorganisms is usually characterized by priming activation (Banani et al., 2013; Conrath et al., 2006; Verhagen et al., 2004) and broad-spectrum effectiveness to various types of pathogens (van der Ent et al., 2009). Therefore, microbial elicitors of induced resistance seems to be promising biocontrol methods for disease management of crops (Vallad and Goodman 2004; Walters et al., 2013). However, the potential of induced resistance has yet to be fully realized, mainly due to its only partial control of the disease and its inconsistency under field conditions (Walters et al., 2005; Walters 2009; Walters et al., 2013). Induced resistance is a plant-mediated mechanism, and its expression is likely to be influenced by a number of factors, including environment, genotype and crop nutrition (Walters et al., 2005; Walters 2009). Activation of defense processes has been found to be negatively affected by limiting nitrogen supply (Dietrich et al., 2004; Walters et al., 2005), and it could also be affected by other abiotic stresses which alter the physiological status of the plant. Concerns about the impact of abiotic stresses on agriculture have been raised in the last decade, especially in light of the predicted effects of climate changes (Lobell et al., 2011). High temperatures and drought associated with heat waves may occur with increased frequency as a result of climate change (Intergovernmental Panel on Climate Change (IPCC) 2007), threatening crop production and influencing interactions with both pathogenic (Eastburn et al., 2011; Pautasso et al., 2012; Yasuda et al., 2008) and beneficial (Compant et al., 2010) microorganisms. Whereas the effects of abiotic stresses on plant resistance to pathogens have been extensively studied (Atkinson and Urwin 2012), their effects on resistance mechanisms activated by resistance inducers has received little attention (Baysal et al., 2007). Moreover, the effects of increased temperature on beneficial plant-associated microorganisms have been found to be variable (Compant et al., 2010), highlighting the complexity of this system.

In this study we investigated the effect of exposing grapevines to a short period of heat and/or drought on plant resistance induced by treatments with the beneficial microorganism T39. To the best of our knowledge, this study is the first to analyze the effect of abiotic stresses on

induced resistance mechanisms in grapevine. We found that exposure to combined heat and drought stresses (HDS) significantly reduced the efficacy of T39-induced resistance to downy mildew. However, the efficacy of T39-induced resistance was not affected by a single abiotic stress (heat or drought). While no symptoms of abiotic stress were visible, Ψ_{leaf} values and expression profiles of heat-stress related genes confirmed that plants reacted to water deficit and to heat. Decrease of Ψ_{leaf} has been previously shown in drought-stressed grapevines (Lovisolo et al., 2002; Lovisolo et al., 2008; Perrone et al., 2012; Pou et al., 2013) and it has been associated with accumulation of ABA and reduction of stomatal conductance (Lovisolo et al., 2002; Pou et al., 2013), confirming activation of drought-stress responses in grapevines at the end of the stressing period. Likewise, modulation of genes encoding heat stress-related proteins (Banilas et al., 2012; Kobayashi et al., 2010; Wahid et al., 2007) indicated that plants reacted to heat, and the combination of abiotic stresses was associated with decreased efficacy of T39 against downy mildew. At the end of the stressing period, the expressions of *PR-2*, *LOX-9* and *OSM-1* were lower in HDS control plants than in NS control plants, demonstrating that abiotic stress negatively affects the basal expression of some defense-related genes. Negative effects of abiotic stresses on gene expression were also found during T39-induced resistance. The expression levels of *PR-2*, *LOX-9* and *OSM-1* were lower in HDS T39-treated plants than in NS T39-treated plants before inoculation, indicating attenuated reaction to the beneficial microorganism in stressed plants. In HDS T39-treated plants, no priming of *PR-2*, *OSM-1* and *CHIT3* was observed and expression of *PR-2*, *LOX-9* and *OSM-1* after *P. viticola* inoculation was attenuated, in agreement with the weaker efficacy of T39-induced resistance. However, the expression levels of *PR-4*, *CHIT3* and *ERF* after inoculation were comparable in HDS and NS plants, indicating that abiotic stresses did not cause global inhibition of transcription for all genes related to T39-induced resistance.

The abiotic stress response in plants is mainly controlled by ABA (Cramer et al., 2011), and this hormone is also involved in regulating defense responses to biotic stresses together with the SA, JA and ET signaling pathways (Robert-Seilaniantz et al., 2011). Crosstalk of the signaling pathways that tune plant reactions to biotic and abiotic stresses is extremely complex, and ABA signaling plays a crucial role in modulating defense reactions under exposure to multiple stresses (Asselbergh et al., 2008; Atkinson and Urwin 2012; Robert-Seilaniantz et al., 2011; Ton et al., 2009). Recent evidences have highlighted that plants respond differently to

multiple stresses than to individual stresses (Atkinson and Urwin 2012), confirming that more information is needed on the effect of abiotic stresses on biocontrol tools based on induced resistance (Walters et al., 2005). For example, it has been shown that salt stress has a positive effect on β-aminobutyric acid (BABA)-induced resistance (Baysal et al., 2007). However, ABA signaling mediates both BABA-induced resistance (Ton and Mauch-Mani 2004) and tolerance to salt stress (Achard et al., 2006; Duan et al., 2013), suggesting synergic activation of ABA signaling in response to abiotic stress and BABA treatment. Our results show that abiotic stresses antagonize T39-induced resistance to downy mildew in grapevine. T39-induced resistance is mediated by JA and ET signaling pathways (Korolev et al., 2008; Perazzolli et al., 2011) and it was not efficiently activated in ABA-impaired *Arabidopsis* mutants (Korolev et al., 2008), indicating crosstalk in hormonal signaling during T39-induced resistance. ABA signaling antagonizes the JA- and ET-mediated defense response (Anderson et al., 2004; Atkinson and Urwin 2012), in agreement with the negative effects of abiotic stresses on T39-induced resistance. Therefore, the effects of abiotic stresses on induced resistance are highly dependent on the resistance inducer and the signaling molecules involved, demonstrating the need for specific characterizations for each inducer and each pathosystem.

Although resistance inducers represent potential biocontrol alternatives to chemical fungicides given their low impact for the environment, more knowledge about the role of the physiological status of the plant is required in order to maximize their efficacy in field conditions (Walters et al., 2013). The evidence reported here indicated that exposure to abiotic stresses could be one of the possible explanations for the inconsistencies and weaknesses of resistance inducers in field conditions, where plants are frequently exposed to various abiotic stresses. This information seems to be particularly important given the future climate changes, since warmer and drier summers are predicted in several regions (Intergovernmental Panel on Climate Change (IPCC) 2007). In order to further analyze the effect of resistance inducers under filed conditions, the possible exposure of plants to abiotic stresses need to be taken into account in future experimental trials. For this purpose, expression analysis of defense-related genes and abiotic stress markers in vineyards could be helpful to evaluate the plant status and to predict the ability of the plant to react to resistance inducers. Accurate studies investigating the thresholds of abiotic stresses (alone or in combination) which affect crop responses to beneficial and pathogenic microorganisms need to be carried out, and the expression analysis of specific

defense-related genes will contribute to the quantitative analysis of the impact of abiotic conditions on induced resistance under varying environmental conditions.

ACKNOWLEDGEMENTS

This research was supported by the EnviroChange Project funded by the Autonomous Province of Trento and the EU project CO-FREE (theme KBBE.2011.1.2-06, grant agreement number 289497). The authors thank Oscar Giovannini for technical support with the greenhouse experiments and Dr. Roberto Zorer for providing us with the Scholander-type pressure chamber.

REFERENCES

Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., Van Der Straeten, D., Peng, J. and Harberd, N. P. (2006). Integration of plant responses to environmentally activated phytohormonal signals. Science. 5757: 91-94

Adie, B. A. T., Pérez-Pérez, J., Pérez-Pérez, M. M., Godoy, M., Sánchez-Serrano, J.-J., Schmelz, E. A. and Solano, R. (2007). ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. Plant Cell. 5: 1665-1681

Anderson, J. P., Badruzsaufari, E., Schenk, P. M., Manners, J. M., Desmond, O. J., Ehlert, C., Maclean, D. J., Ebert, P. R. and Kazan, K. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. Plant Cell. 12: 3460-3479

Asselbergh, B., De Vleesschauwer, D. and Höfte, M. (2008). Global switches and fine-tuning-ABA modulates plant pathogen defense. Mol. Plant-Microbe Interact. 6: 709-719

Atkinson, N. J. and Urwin, P. E. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. J. Exp. Bot. 10: 3523-3543

Banani, H., Roatti, B., Ezzahi, B., Giovannini, O., Gessler, C., Pertot, I. and Perazzolli, M. (2013). Characterization of resistance mechanisms activated by *Trichoderma harzianum* T39 and benzothiadiazole to downy mildew in different grapevine cultivars. Plant Pathology. Accepted

Banilas, G., Korkas, E., Englezos, V., Nisiotou, A. A. and Hatzopoulos, P. (2012). Genome-wide analysis of the heat shock protein 90 gene family in grapevine (*Vitis vinifera* L.). Aust. J. Grape Wine Res. 1: 29-38

Baysal, Ö., Gürsoy, Y. Z., Örnek, H., Çetinel, B. and Teixeira da Silva, J. A. (2007). Enhanced systemic resistance to bacterial speck disease caused by *Pseudomonas syringae* pv. tomato by DL-β-aminobutyric acid under salt stress. Physiol. Plant. 3: 493-506

Casagrande, K., Falginella, L., Castellarin, S., Testolin, R. and Di Gaspero, G. (2011). Defence responses in *Rpv3*-dependent resistance to grapevine downy mildew. Planta. 6: 1097-1109

Ciais, P., Reichstein, M., Viovy, N., Granier, A., Ogee, J., Allard, V., Aubinet, M., Buchmann, N., Bernhofer, C., Carrara, A., Chevallier, F., De Noblet, N., Friend, A. D., Friedlingstein, P., Grunwald, T., Heinesch, B., Keronen, P., Knohl, A., Krinner, G., Loustau, D., Manca, G., Matteucci, G., Miglietta, F., Ourcival, J. M., Papale, D., Pilegaard, K., Rambal, S., Seufert, G., Soussana, J. F., Sanz, M. J., Schulze, E. D., Vesala, T. and Valentini, R. (2005). Europe-wide reduction in primary productivity caused by the heat and drought in 2003. Nature. 7058: 529-533

Compant, S., Van Der Heijden, M. G. A. and Sessitsch, A. (2010). Climate change effects on beneficial plant–microorganism interactions. FEMS Microbiol. Ecol. 2: 197-214

Conrath, U., Beckers, G. J. M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M.-A., Pieterse, C. M. J., Poinssot, B., Pozo, M. J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L. and Mauch-Mani, B. (2006). Priming: getting ready for battle. Mol. Plant-Microbe Interact. 10: 1062-1071

Corio-Costet, M. F., Dufour, M. C., Cigna, J., Abadie, P. and Chen, W. J. (2011). Diversity and fitness of *Plasmopara viticola* isolates resistant to QoI fungicides. Eur. J. Plant Pathol. 2: 315-329

Cramer, G., Urano, K., Delrot, S., Pezzotti, M. and Shinozaki, K. (2011). Effects of abiotic stress on plants: a systems biology perspective. BMC Plant Biol. 1: 163

Dietrich, R., Ploß, K. and Heil, M. (2004). Constitutive and induced resistance to pathogens in *Arabidopsis thaliana* depends on nitrogen supply. Plant Cell Environ. 7: 896-906

Duan, L., Dietrich, D., Ng, C. H., Chan, P. M. Y., Bhalerao, R., Bennett, M. J. and Dinneny, J. R. (2013). Endodermal ABA signaling promotes lateral root quiescence during salt stress in *Arabidopsis* seedlings. Plant Cell. 1: 324-341

Eastburn, D. M., McElrone, A. J. and Bilgin, D. D. (2011). Influence of atmospheric and climatic change on plant–pathogen interactions. Plant Pathol. 1: 54-69

European and Mediterranean Plant Protection Organization (EPPO) (2001). Guidelines for the efficacy evaluation of fungicides: *Plasmopara viticola*. EPPO Bulletin. 31: 313-317

Flexas, J., Barón, M., Bota, J., Ducruet, J. M., Gallé, A., Galmés, J., Jiménez, M., Pou, A., Ribas-Carbó, M., Sajnani, C., Tomàs, M. and Medrano, H. (2009). Photosynthesis limitations during water stress acclimation and recovery in the drought-adapted *Vitis* hybrid Richter-110 (*V. berlandieri*×*V. rupestris*). J. Exp. Bot. 8: 2361-2377

Flors, V., Ton, J., Van Doorn, R., Jakab, G., García-Agustín, P. and Mauch-Mani, B. (2008). Interplay between JA, SA and ABA signalling during basal and induced resistance against *Pseudomonas syringae* and *Alternaria brassicicola*. Plant J. 1: 81-92

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr. Opin. Plant Biol. 4: 436-442

Gessler, C., Pertot, I. and Perazzolli, M. (2011). *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. Phytopathol. Mediterr. 3-44

Hamiduzzaman, M. M., Jakab, G., Barnavon, L., Neuhaus, J. M. and Mauch-Mani, B. (2005). β-aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. Mol. Plant-Microbe Interact. 8: 819-829

Intergovernmental Panel on Climate Change (IPCC) (2007). Climate change. Synthesis report. Summary for policymarkers. Available at http://www.ipcc.ch.

Kobayashi, M., Katoh, H., Takayanagi, T. and Suzuki, S. (2010). Characterization of thermotolerance-related genes in grapevine (*Vitis vinifera*). J. Plant Physiol. 10: 812-819

Korolev, N., Rav David, D. and Elad, Y. (2008). The role of phytohormones in basal resistance and *Trichoderma*-induced systemic resistance to *Botrytis cinerea* in *Arabidopsis thaliana*. BioControl. 4: 667-683

Liu, G. T., Wang, J. F., Cramer, G., Dai, Z. W., Duan, W., Xu, H. G., Wu, B. H., Fan, P. G., Wang, L. J. and Li, S. H. (2012). Transcriptomic analysis of grape (*Vitis vinifera* L.) leaves during and after recovery from heat stress. BMC Plant Biol. 174

Lobell, D. B., Schlenker, W. and Costa-Roberts, J. (2011). Climate trends and global crop production since 1980. Science. 6042: 616-620

Longa, C. M. O., Savazzini, F., Tosi, S., Elad, Y. and Pertot, I. (2009). Evaluating the survival and environmental fate of the biocontrol agent *Trichoderma atroviride* SC1 in vineyards in northern Italy. J. Appl. Microbiol. 5: 1549-1557

Lovisolo, C., Hartung, W. and Schubert, A. (2002). Whole-plant hydraulic conductance and root-to-shoot flow of abscisic acid are independently affected by water stress in grapevines. Funct. Plant Biol. 11: 1349-1356

Lovisolo, C., Perrone, I., Hartung, W. and Schubert, A. (2008). An abscisic acid-related reduced transpiration promotes gradual embolism repair when grapevines are rehydrated after drought. New Phytol. 3: 642-651

Malacarne, G., Vrhovsek, U., Zulini, L., Cestaro, A., Stefanini, M., Mattivi, F., Delledonne, M., Velasco, R. and Moser, C. (2011). Resistance to *Plasmopara viticola* in a grapevine segregating population is associated with stilbenoid accumulation and with specific host transcriptional responses. BMC Plant Biol. 1: 114

Mauch-Mani, B. and Mauch, F. (2005). The role of abscisic acid in plant–pathogen interactions. Curr. Opin. Plant Biol. 4: 409-414

Palmieri, M. C., Perazzolli, M., Matafora, V., Moretto, M., Bachi, A. and Pertot, I. (2012). Proteomic analysis of grapevine resistance induced by *Trichoderma harzianum* T39 reveals specific defence pathways activated against downy mildew. J. Exp. Bot. 17: 6237-6251

Pautasso, M., Döring, T., Garbelotto, M., Pellis, L. and Jeger, M. (2012). Impacts of climate change on plant diseases-opinions and trends. Eur. J. Plant Pathol. 1: 295-313

Perazzolli, M., Bampi, F., Faccin, S., Moser, M., De Luca, F., Ciccotti, A. M., Velasco, R., Gessler, C., Pertot, I. and Moser, C. (2010). *Armillaria mellea* induces a set of defense genes in grapevine roots and one of them codifies a protein with antifungal activity. Mol. Plant-Microbe Interact. 4: 485-496

Perazzolli, M., Dagostin, S., Ferrari, A., Elad, Y. and Pertot, I. (2008). Induction of systemic resistance against *Plasmopara viticola* in grapevine by *Trichoderma harzianum* T39 and benzothiadiazole. Biol. Control. 2: 228-234

Perazzolli, M., Moretto, M., Fontana, P., Ferrarini, A., Velasco, R., Moser, C., Delledonne, M. and Pertot, I. (2012). Downy mildew resistance induced by *Trichoderma harzianum* T39 in susceptible grapevines partially mimics transcriptional changes of resistant genotypes. BMC Genomics. 1: 660

Perazzolli, M., Roatti, B., Bozza, E. and Pertot, I. (2011). *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defense without costs for grapevine. Biol. Control. 1: 74-82

Perrone, I., Pagliarani, C., Lovisolo, C., Chitarra, W., Roman, F. and Schubert, A. (2012). Recovery from water stress affects grape leaf petiole transcriptome. Planta. 6: 1383-1396

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 9: e45

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S. and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. Nat. Chem. Biol. 5: 308-316

Pieterse, C. M. J., van der Does, D., Zamioudis, C., Leon-Reyes, A. and van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. Annu. Rev. Cell Dev. Biol. 1: 489-521

Pieterse, C. M. J., Van Pelt, J. A., Ton, J., Parchmann, S., Mueller, M. J., Buchala, A. J., Métraux, J. P. and Van Loon, L. C. (2000). Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. Physiol. Mol. Plant Pathol. 3: 123-134

Polesani, M., Bortesi, L., Ferrarini, A., Zamboni, A., Fasoli, M., Zadra, C., Lovato, A., Pezzotti, M., Delledonne, M. and Polverari, A. (2010). General and species-specific transcriptional

responses to downy mildew infection in a susceptible (*Vitis vinifera*) and a resistant (*V. riparia*) grapevine species. BMC Genomics. 1: 117

Poni, S., Bernizzoni, F., Civardi, S., Gatti, M., Porro, D. and Camin, F. (2009). Performance and water-use efficiency (single-leaf vs. whole-canopy) of well-watered and half-stressed split-root Lambrusco grapevines grown in Po Valley (Italy). Agric. Ecosyst. Environ. 1–3: 97-106

Pou, A., Medrano, H., Flexas, J. and Tyerman, S. D. (2013). A putative role for TIP and PIP aquaporins in dynamics of leaf hydraulic and stomatal conductances in grapevine under water stress and re-watering. Plant Cell Environ. 4: 828-843

Robert-Seilaniantz, A., Grant, M. and Jones, J. D. G. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Annu. Rev. Phytopathol. 1: 317-343

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B. and Moorman, A. F. M. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 6: e45

Shoresh, M., Harman, G. E. and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. Annu. Rev. Phytopathol. 1: 21-43

Steele, M. R., Gitelson, A. A. and Rundquist, D. C. (2008). A comparison of two techniques for nondestructive measurement of chlorophyll content in grapevine leaves. Agron. J. 3: 779-782

Ton, J., Flors, V. and Mauch-Mani, B. (2009). The multifaceted role of ABA in disease resistance. Trends Plant Sci. 6: 310-317

Ton, J. and Mauch-Mani, B. (2004). β-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J. 1: 119-130

Trouvelot, S., Varnier, A. L., Allègre, M., Mercier, L., Baillieul, F., Arnould, C., Gianinazzi-Pearson, V., Klarzynski, O., Joubert, J. M., Pugin, A. and Daire, X. (2008). A β-1,3 glucan sulfate induces resistance in grapevine against *Plasmopara viticola* through priming of defense responses, including HR-like cell death. Mol. Plant-Microbe Interact. 2: 232-243

Vallad, G. E. and Goodman, R. M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. Crop Sci. 6: 1920-1934

van der Ent, S., Koornneef, A., Ton, J. and Pieterse, C. M. J. (2009) Induced resistance – Orchestrating defence mechanisms through crosstalk and priming. In: Annual Plant Reviews Volume 34: Molecular Aspects of Plant Disease Resistance, Wiley-Blackwell, Oxford, UK., 334-370

Velasco, R., Zharkikh, A., Troggio, M., Cartwright, D. A., Cestaro, A., Pruss, D., Pindo, M., FitzGerald, L. M., Vezzulli, S., Reid, J., Malacarne, G., Iliev, D., Coppola, G., Wardell, B., Micheletti, D., Macalma, T., Facci, M., Mitchell, J. T., Perazzolli, M., Eldredge, G., Gatto, P., Oyzerski, R., Moretto, M., Gutin, N., Stefanini, M., Chen, Y., Segala, C., Davenport, C., Demattè, L., Mraz, A., Battilana, J., Stormo, K., Costa, F., Tao, Q., Si-Ammour, A., Harkins, T., Lackey, A., Perbost, C., Taillon, B., Stella, A., Solovyev, V., Fawcett, J. A., Sterck, L., Vandepoele, K., Grando, S. M., Toppo, S., Moser, C., Lanchbury, J., Bogden, R., Skolnick, M., Sgaramella, V., Bhatnagar, S. K., Fontana, P., Gutin, A., Van de Peer, Y., Salamini, F. and Viola, R. (2007). A high quality draft

consensus sequence of the genome of a heterozygous grapevine variety. PLoS ONE. 12: e1326
Verhagen, B. W. M., Glazebrook, J., Zhu, T., Chang, H.-S., van Loon, L. C. and Pieterse, C. M. J. (2004). The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. Mol. Plant-Microbe Interact. 8: 895-908
Wahid, A., Gelani, S., Ashraf, M. and Foolad, M. R. (2007). Heat tolerance in plants: an overview. Environ. Exp. Bot. 3: 199-223
Walters, D., Walsh, D., Newton, A. and Lyon, G. (2005). Induced resistance for plant disease control: maximizing the efficacy of resistance elicitors. Phytopathology. 12: 1368-1373
Walters, D. R. (2009). Are plants in the field already induced? Implications for practical disease control. Crop Prot. 6: 459-465
Walters, D. R., Ratsep, J. and Havis, N. D. (2013). Controlling crop diseases using induced resistance: challenges for the future. J. Exp. Bot. 5: 1263-1280
Yasuda, M., Ishikawa, A., Jikumaru, Y., Seki, M., Umezawa, T., Asami, T., Maruyama-Nakashita, A., Kudo, T., Shinozaki, K., Yoshida, S. and Nakashita, H. (2008). Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in *Arabidopsis*. Plant Cell. 6: 1678-1692

SUPPLEMENTALS

Supplemental table 1. Downy mildew severity in plants exposed to unstressed (NS), drought-stressed (DS), heat-stressed (HS), and heat + drought stressed (HDS) conditions and treated with distilled water (Control) or *Trichoderma harzianum* T39 (T39).

Growth condition	Downy mildew severity (%)			
	Control		T39-treated	
NS	64.2 ± 9.2	a	34.1 ± 9.1	b
DS	64.4 ± 8.8	a	36.2 ± 7.2	b
HS	63.5 ± 10.4	a	32.2 ± 8.8	b
HDS	73.2 ± 4.3	a	59.5 ± 4.5	a

Downy mildew severity was assessed on all leaves of each plant as percentage of abaxial leaf area covered by sporulation of downy mildew. F-test revealed non-significant treatment-experiment interactions ($P > 0.05$) and data from two repetitions of the experiment were pooled. Mean severity scores and standard errors were calculated from 12 replicates (plants) per treatment pooled from two independent repetitions of the experiment. Different letters indicate significant differences among treatments and growth conditions, according to Fisher's test ($P < 0.05$).

Chapter 4

Characterization of resistance mechanisms activated by *Trichoderma harzianum* T39 and benzothiadiazole to downy mildew in different grapevine cultivars

Houda Banani[+], Benedetta Roatti[+], Bahcine Ezzahi, Gesare Gessler, Ilaria Pertot and Michele Perazzolli.

[+]These authors contributed equally to this work

Accepted for publication on May 17th (2013) in Plant Pathology: DOI:10.1111/ppa.12089

*Benedetta Roatti contributed to the sections on real-time RT-PCR, sequences information, data analysis and wrote the paper.

ABSTRACT

Downy mildew, caused by *Plasmopara viticola*, is one of the most destructive diseases of grapevine and is controlled with intense application of chemical fungicides. Treatment with *Trichoderma harzianum* T39 (T39) or benzothiadiazole-7-carbothioic acid S-methyl ester (BTH) has been previously shown to activate grapevine resistance to downy mildew and reduce disease symptoms in the Pinot noir cultivar. However, enhancement of plant resistance can be affected by several factors, including plant genotype. In order to further extend the use of resistance inducers against downy mildew, we characterized the physiological and molecular properties of T39- and BTH-activated resistance in different cultivars of table and wine grapes under greenhouse conditions. T39 treatment reduced downy mildew symptoms, but the degree of efficacy differed significantly among grapevine cultivars. However, efficacy of BTH-activated resistance was consistently high in the different cultivars. Expression profiles of defence-related genes differed among cultivars in response to resistance inducers and to pathogen inoculation. T39 treatment enhanced the expression of defence-related genes in the responsive cultivars, before and after *P. viticola* inoculation. A positive correlation between the efficacy of T39 and the expression level of defence-related genes was found in Primitivo and Pinot noir plants, while different genes or more complex processes were probably activated in Sugraone and Negroamaro. The data reported here suggests that the use of a responsive cultivar is particularly important to maximize the efficacy of resistance inducers and new natural inducers should be explored for the less responsive cultivars.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the world's major fruit crops, but most of the commercial cultivars used for table grape or wine production are susceptible to downy mildew. Grapevine downy mildew, caused by the oomycete *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni, is a devastating disease, particularly in warm and wet climates (Gessler et al., 2011). *P. viticola* attacks leaves and young berries and is controlled with frequent applications of chemical fungicides to avoid yield losses and reduced berry quality. Concerns about the environmental impact of the overuse of pesticides have sparked interest in developing alternative methods to chemical treatments (Gessler et al., 2011). Therefore, a large number of researchers are currently engaged in identifying efficient biocontrol agents to limit downy mildew infection in susceptible cultivars (Harm et al., 2011).

Several molecules have been shown to increase resistance to downy mildew in susceptible grapevines, such as chitosan (Aziz et al., 2006), laminarin (Aziz et al., 2003), sulfated laminarin (Steimetz et al., 2012; Trouvelot et al., 2008), oligogalacturonide (Allègre et al., 2009), β-aminobutyric acid (BABA) (Hamiduzzaman et al., 2005), fosetyl-aluminium (Dercks and Creasy 1989), thiamine (Boubakri et al., 2012) and benzothiadiazole-7-carbothioic acid S-methyl ester (BTH) (Perazzolli et al., 2008), as well as combinations of different resistance inducers (Pinto et al., 2012; Reuveni et al., 2001). Application of plant extracts or microbial agents can also induce resistance to downy mildew in grapevine, for example *Rheum palmatum* (Godard et al., 2009) and *Solidago canadensis* (Harm et al., 2011) plant extracts, organic amendments (Thuerig et al., 2011), the beneficial microorganisms *Aureobasidium pullulans* (Harm et al., 2011) and *Trichoderma harzianum* T39 (T39) (Perazzolli et al., 2008).

Enhancement of plant resistance exploits mechanisms of the plant immune system and two phenotypically similar forms of systemic immunity have so far been identified in plants: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Pieterse et al., 2009). SAR is controlled by SA-dependent signalling pathways and is activated systemically following pathogen recognition or treatment with some chemicals (Pieterse et al., 2009). ISR can be activated by specific strains of beneficial microorganisms and is usually regulated by jasmonic acid (JA) and ethylene (ET) signals (Pieterse et al., 2009). ISR is characterized by broad-spectrum activity against various types of pathogens and abiotic stresses and usually involves the

activation of a priming state (van der Ent et al., 2009). Primed plants display faster and/or more intense activation of the defence responses after pathogen inoculation, and this mechanism provides advantages in terms of energy costs under pathogen-free conditions, as defences are activated only when they are really needed (Conrath et al., 2006).

In grapevine, T39 and BTH significantly reduce downy mildew symptoms, both locally and systemically, without any direct toxic effects on *P. viticola* sporangia, indicating that the control mechanisms are mainly related to the activation of plant resistance (Perazzolli et al., 2008). Different mechanisms are activated by BTH and T39, making these resistance inducers useful tools for comparative characterization of resistance activation in grapevine (Palmieri et al., 2012; Perazzolli et al., 2011). BTH treatment directly induced the expression of SA-regulated pathogenesis-related (*PR*) genes (Dufour et al., 2012; Perazzolli et al., 2011), caused direct accumulation of reactive oxygen species (ROS) (Palmieri et al., 2012) and antimicrobial stilbenes (Dufour et al., 2012). T39 treatment produced a dual effect: it directly activated the microbial recognition machinery and enhanced the expression of defence-related processes after downy mildew inoculation in the Pinot noir cultivar (Palmieri et al., 2012; Perazzolli et al., 2012). T39 treatment did not entail any apparent energy costs (Perazzolli et al., 2011), suggesting low risk to grape production. Activation of plant defences appears to be a promising low-impact tool for controlling crop diseases (Vallad and Goodman 2004; Walters et al., 2012). However, this method is currently far from being widely used because resistance inducers rarely provide complete disease control and their effects are often inconsistent in field conditions (Harm et al., 2011; Walters et al., 2012; Walters et al., 2013). Since induced resistance is a host response, it is likely to be affected by the environment in field conditions (Thuerig et al., 2011; Walters et al., 2013) and by plant genotype (Reuveni et al., 2001; Sharma et al., 2010; Tucci et al., 2011; Walters et al., 2011). Greater understanding of these complex interactions is therefore required in order to maximize the efficacy of resistance inducers in the field (Walters et al., 2012; Walters et al., 2013).

In this study, we investigated the physiological and molecular mechanisms activated by T39 and BTH against downy mildew in different grapevine cultivars under controlled greenhouse conditions. The efficacy of BTH-activated resistance was consistent among the grapevine cultivars tested, but there were differences in the expression profiles of defence-related genes. Efficacy of T39-induced resistance differed significantly among cultivars and gene

expression results indicated that more complex mechanisms are probably involved in the highly responsive cultivars.

MATERIALS AND METHODS

Plant material

Rooted cuttings were obtained from seven wine grape cultivars: Pinot noir (PNR), Pinot gris (PGR), Chardonnay (CHR), Merlot (MER), Negroamaro (NEG), Primitivo (PRI) and Uva di Troia (UVA); and from seven table grape cultivars: Italia (ITA), Crimson seedless (CRM), Black magic (BLM), Sugraone (SUG), Victoria (VIC), Red Globe (RED) and Michele Palieri (MIC; Table 1). Cuttings were planted in 2.5 L pots (three cuttings in each pot) containing a mixture of peat and pumice (3:1) and grown for two months under greenhouse conditions at 25 ± 1 °C with a photoperiod of 16 h of light and a relative humidity (RH) of 60 ± 10%, until each plant had one shoot with five to ten leaves.

Table 1 Grapevine cultivars.

Grapevine cultivars [a]		Cultivar abbreviation	Colour of berry skin
Wine grape	Pinot noir	PNR	black
	Pinot gris	PGR	grey
	Chardonnay	CHR	white
	Merlot	MER	black
	Negroamaro	NEG	black
	Primitivo	PRI	black
	Uva di Troia	UVA	black
Table grape	Italia	ITA	white
	Crimson seedless	CRM	red
	Black magic	BLM	black
	Sugraone	SUG	white
	Victoria	VIC	white
	Red Globe	RED	red
	Michele Palieri	MIC	black

[a] Accordingly to the Vitis International Variety Catalogue (www.vivc.de).

Resistance induction in grapevine plants

Resistance activation was obtained using conidia of T39 and the commercial formulation of BTH (Bion, 50WG Syngenta Crop Protection, Basel, Switzerland). To obtain the conidia, T39 mycelium was grown for two weeks at 25°C in the dark on malt extract agar (Oxoid, Basingstoke, U.K.). Five small pieces (3 mm in diameter) of fresh mycelium were used to inoculate twice sterilized rice grains (prepared in Erlenmeyer flasks, containing 20 g of rice and 55 mL of water), which were then incubated at 25°C for three weeks to maximize the yield of vital conidia (Longa et al., 2009). T39 conidia were collected by washing the inoculated rice in cold (4°C) distilled water then filtering the conidia suspension with a fine net. The concentration of the T39 conidia suspension was measured by counting with a haemocytometer under a light microscope. Aqueous suspensions of 1×10^7 T39 conidia mL^{-1} and 0.5 g L^{-1} BTH were used to induce resistance to *P. viticola* (Perazzolli et al., 2011). Control plants were treated with water (H_2O-treated). The abaxial and adaxial surfaces of grapevine leaves were treated three times (one, two and three days before pathogen inoculation) in order to induce the greatest phenotypic resistance activation (Perazzolli et al., 2008). For each treatment, approximately 2 mL per leaf were applied using a compressed-air hand sprayer.

Pathogen inoculation and assessment of disease severity

A *P. viticola* isolate was collected from an untreated vineyard in northern Italy (Trentino region) and maintained by subsequent inoculations on *V. vinifera* Pinot noir plants under greenhouse conditions. To obtain fresh sporangia, infected plants with oil spot symptoms were incubated overnight in the dark at 99-100% RH and 25 ± 1 °C to promote pathogen sporulation. *P. viticola* sporangia were then collected by washing the abaxial surfaces bearing freshly sporulating lesions with cold (4°C) distilled water. The concentration of the inoculum suspension was adjusted to 1×10^5 sporangia mL^{-1} by counting with a haemocytometer under a light microscope. One day after the last treatment with the resistance inducers, approximately 2 mL of the inoculum suspension were applied to the abaxial surface of each leaf using a compressed-air hand sprayer. Inoculated plants were incubated overnight in the dark at 99-100% RH and 25 ± 1 °C and then kept under controlled greenhouse conditions. Six days after inoculation, the plants were incubated overnight in the dark at 25 ± 1 °C with 99-100% RH to promote downy mildew sporulation. Disease severity was visually assessed as the percentage of abaxial leaf area covered

by sporulation, according to standard guidelines of the European and Mediterranean Plant Protection Organization (EPPO 2001). Treatment efficacy was calculated according to the following formula: (disease severity in control plants - disease severity in treated plants)/ disease severity in control plants × 100. Four replicates (pots containing three plants each) per treatment were analysed in a randomized complete block design and two independent repetitions of the experiment were carried out.

Sample collection

Leaf samples were collected immediately before inoculation (uninoculated) and 24 h after *P. viticola* inoculation (inoculated) from H_2O-, T39- and BTH-treated plants. This time point was chosen because it is associated with up-regulation of defence-related genes in Pinot noir plants (Perazzolli et al., 2012; Perazzolli et al., 2011). For each treatment at each time point, leaf samples were collected from three different plants of each cultivar. A total of six plants per treatment were sampled (three before inoculation and three after inoculation) in order to avoid the effects of wounding stress. Each sample comprised two half leaves taken from the same plant; only leaves of the 3rd and 4th node from the top of the shoot were collected in order to select responsive leaves (Steimetz et al., 2012) and to avoid effects of ontogenic resistance (Perazzolli et al., 2008; Thuerig et al., 2011). Samples were immediately frozen in liquid N_2 and stored at -80°C.

Gene expression analysis by quantitative real-time RT-PCR

Four cultivars were selected (Pinot noir, Primitivo, Sugraone and Negroamaro) on the basis of the phenotypic analysis, and gene expression analysis was carried out according to Perazzolli *et al*. (2011). Briefly, total RNA was extracted using the Spectrum Plant total RNA kit (Sigma-Aldrich, St. Louis, MO) and quantified using the NanoDop 8000 (Thermo Fisher Scientific, Wilmington, DE). Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA) and the first strand cDNA was synthesized from 1.0 μg of total RNA using Superscript III (Invitrogen) and oligo-dT. Reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and specific primers (Table 2) using the Light Cycler 480 (Roche Diagnostics, Germany). The PCR conditions were: 50°C for 2 min and 95°C for 2 min as initial steps, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was examined in

Table 2 Primer sequences for quantitative real-time RT-PCR expression analysis of grapevine genes.

Gene name	Gene code[a]	Primer sequence[b]	
Pathogenesis-related protein 1 (*PR-1*)	fgenesh.VV78X169899.6_1	*PR-1 for* *PR-1 rev*	ACTTGTGGGTGGGGGAGAA TGTTGCATTGAACCCTAGCG
Pathogenesis-related protein 2 (*PR-2*)	twinscan.VV78X005385.7_1	*PR-2 for* *PR-2 rev*	GTTATTTCAGAGAGTGGTTGGC AACATGGCAAACACGTAAGTCT
Pathogenesis-related protein 4 (*PR-4*)	glimmer.VV78X053121.6_1	*PR-4 for* *PR-4 rev*	CAGGCAACGGTGAGAATAGT ACCACAGTCCACAAACTCGTA
Osmotin 1 (*OSM-1*)	glimmer.VV78X132476.3_2	*OSM-1 for* *OSM-1 rev*	CGCTGCGCTAAAGACTACC AAAAACCTTGAGTAATCTGTAGCA
Actin	glimmer.VV78X114914.6_2	*ACT for* *ACT rev*	ATTCCTCACCATCATCAGCA GACCCCCTCCTACTAAAACT

[a] The corresponding Pinot Noir genes used for primer design and sequences alignments were identified on the *Vitis vinifera* Pinot Noir predicted genes (Velasco et al., 2007) of the release 3 (http://genomics.research.iasma.it/).
[b] Primer pairs: *PR-1*, *PR-2*, *PR-4* and *Actin* (Perazzolli et al., 2010; Perazzolli et al., 2012; Perazzolli et al., 2011; Polesani et al., 2010).

three technical replicates and dissociation curves were analysed to verify the specificity of each amplification reaction. To validate amplification of the target genes, real-time PCR products were sequenced on both strands using the Sanger method and amplified sequences of the different cultivars were aligned by ClustalW2 tool (www.ebi.ac.uk) and compared with the corresponding grapevine Pinot noir predicted gene (Velasco et al., 2007) of Release 3 (http://genomics.research.iasma.it/).

Cycle-threshold (Ct) values were obtained by second derivative calculation using Light Cycler 480 SV1.5.0 software (Roche), and reaction efficiencies were calculated with LinReg software (Ruijter et al., 2009). The *Actin* gene was used as the constitutive gene for normalization because its expression was not significantly affected by the treatments (Perazzolli et al., 2012). The relative expression of each gene was calculated according to the Pfaffl equation (Pfaffl 2001) using uninoculated H_2O-treated Pinot noir plants as calibrator. The Pinot noir cultivar was selected as reference for data calibration, based on our previous gene expression analysis (Perazzolli et al., 2012; Perazzolli et al., 2011) and physiological characterization of T39- and BTH- activated resistance (Palmieri et al., 2012; Perazzolli et al., 2008). The priming effect was calculated as the ratio between the expression level in inoculated leaves of plants treated with the resistance inducer and the expression level in inoculated leaves of H_2O-treated plants, using a threshold of 1.5-fold to identify augmented expressions (Verhagen et al., 2004). Mean expressions and standard errors of three biological replicates (plants) were calculated for each sample, and two independent repetitions of the experiment were analysed.

Statistical analysis

The results of the two independent experiments were analysed. Data were analysed using Statistica 9 software (StatSoft, Tulsa, OK). F-test revealed non-significant treatment-experiment interactions ($P > 0.05$) so the data from the two experiments were pooled. Disease severity and efficacy scores were normalized (K-S test, $P > 0.05$) by root square transformation, while fold change values of gene expression analysis were transformed using the equation $Y = \log_{10}(1 + x)$ (Casagrande et al., 2011). Following validation of variance homogeneity (Levene's test, $P > 0.05$), an analysis of variance (ANOVA) was carried out using Fisher's test to detect significant differences ($P < 0.05$) among cultivars.

RESULTS

Efficacy of induced resistance in the grapevine cultivars

The degree of downy mildew severity in ten different *V. vinifera* cultivars was comparable to the Pinot noir cultivar (Fig. 1a), which was used as the reference for ISR characterization, based on our previous results (Palmieri et al., 2012; Perazzolli et al., 2008; Perazzolli et al., 2012; Perazzolli et al., 2011). The disease severity was lower in Negroamaro and Crimson seedless plants than in Pinot noir and in the highly susceptible Primitivo and Michele Palieri cultivars. Moreover, disease severity was lower in the Black magic than in the other *V. vinifera* cultivars, suggesting a higher level of basal resistance.

Treatment with T39 conidia significantly reduced downy mildew symptoms in the susceptible cultivar Pinot noir (Fig. 1b), in agreement with our previous findings obtained with a commercial formulation of T39 (Perazzolli et al., 2008; Perazzolli et al., 2012; Perazzolli et al., 2011). Although T39-induced resistance reduced downy mildew symptoms in all cultivars tested, there were significant differences in the degree of efficacy. In particular, T39 efficacy was higher in Negroamaro and Black magic than in the reference cultivar Pinot noir, but it was lower in Primitivo than in Pinot noir, suggesting that this mechanism is affected by the plant genotype. On the other hand, BTH treatment activated a consistently high level of resistance to downy mildew with no differences among the cultivars tested (Fig. 1c).

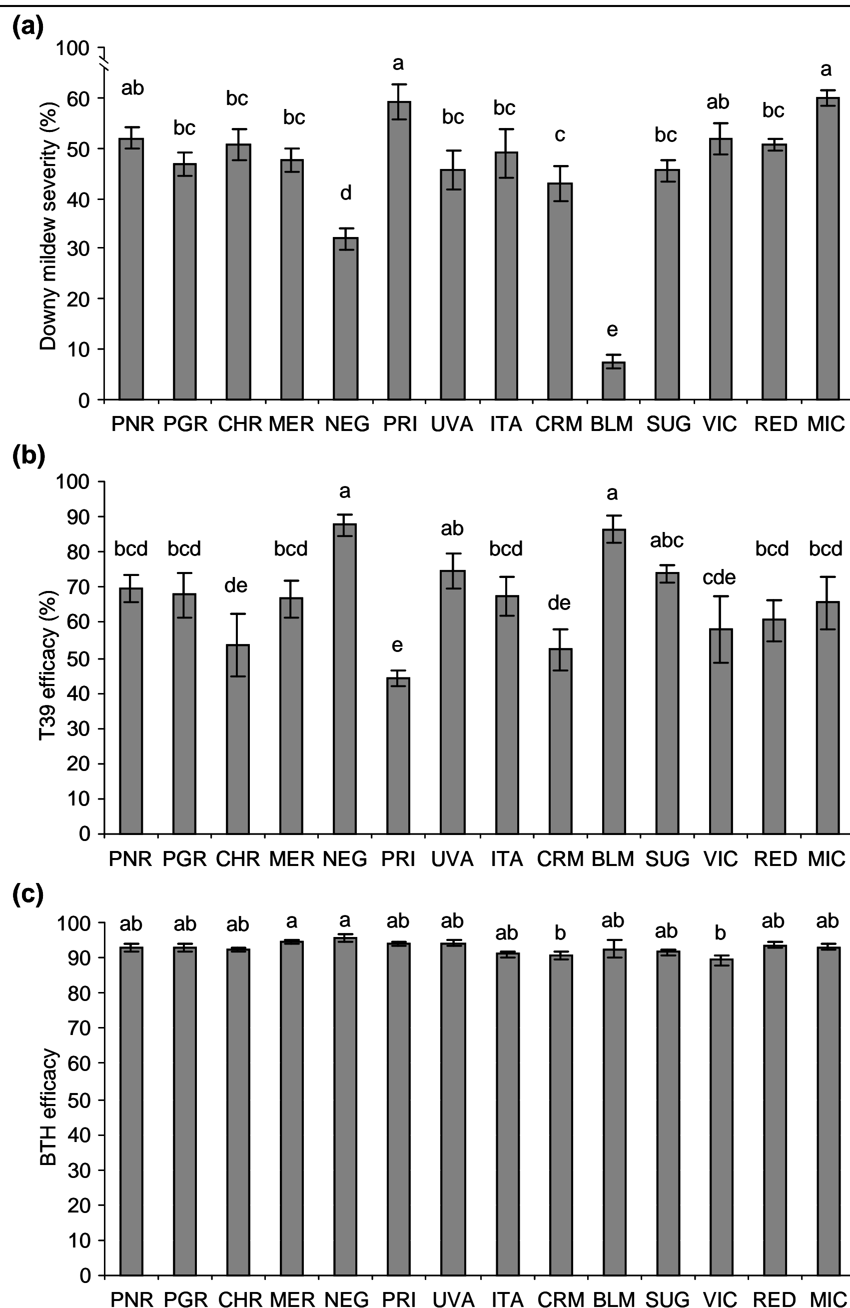

Figure 1. Downy mildew severity in control plants (H_2O-treated; **a**), efficacy of *Trichoderma harzianum* T39 (T39; **b**) and efficacy of benzothiadiazole (BTH; **c**) on Pinot noir (PNR), Pinot gris (PGR), Chardonnay (CHR), Merlot (MER), Negroamaro (NEG), Primitivo (PRI), Uva di Troia (UVA), Italia (ITA), Crimson seedless (CRM), Black magic (BLM), Sugraone (SUG), Victoria (VIC), RedGlobe (RED) and Michele Palieri (MIC) cultivars. Disease severity was assessed as percentage of abaxial leaf area covered by sporulation. Efficacy of T39 and BTH were calculated for each cultivar accordingly to the formula: (disease severity in control plants - disease severity in treated plants)/disease severity in control plants × 100. F-test revealed non-significant treatment-experiment interactions ($P > 0.05$) and data from two repetitions of the experiment were pooled. The mean scores and standard errors of eight replicates (eight pots each containing three plants) pooled from the two experiments are presented for each cultivar. For each panel, different letters indicate significant differences among cultivars according to Fisher's test ($P < 0.05$).

Molecular mechanisms of induced resistance in the grapevine cultivars

In order to analyse the molecular mechanisms involved in T39-induced resistance in grapevine cultivars, gene expression analysis was carried on plants exhibiting lowest (Primitivo) and highest (Negroamaro) levels of T39 efficacy. Gene expression profiles were also analyzed in a table grape cultivar (Sugraone) with a level of T39-efficacy comparable to the wine grape cultivar (Pinot noir), used as reference for ISR characterization (Perazzolli et al., 2012; Perazzolli et al., 2011).

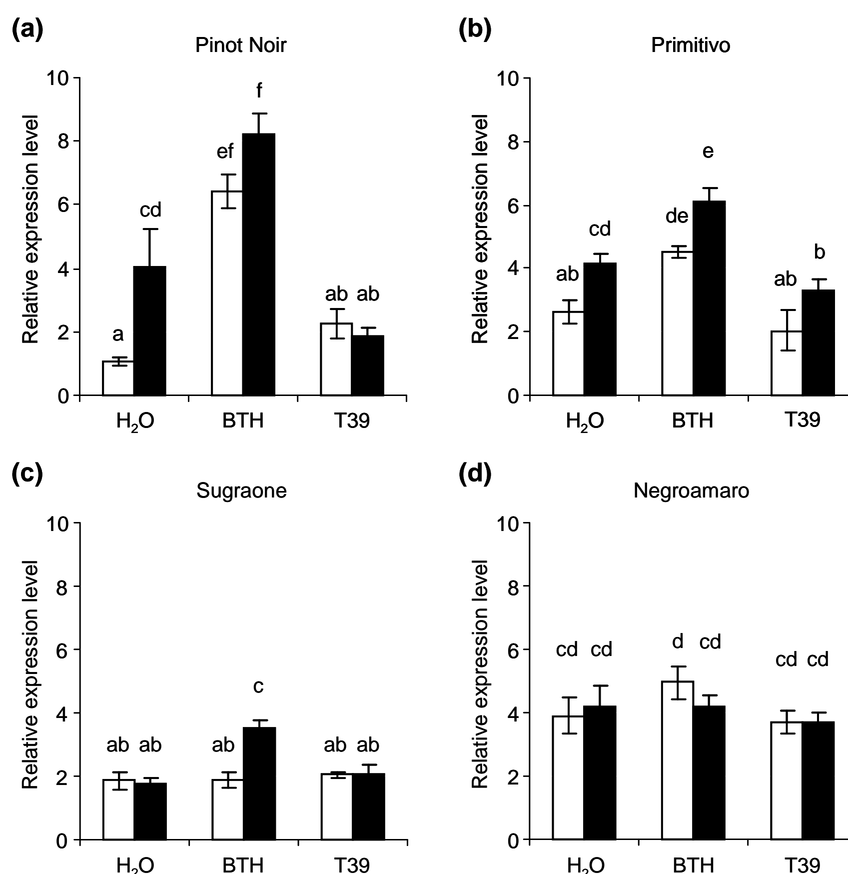

Figure 2. Expression of the defence gene encoding the pathogenesis-related protein 1 (*PR-1*) was analysed in leaf samples collected immediately before (white) and 24 h after (black) *Plasmopara viticola* inoculation of leaves of Pinot noir (**a**), Primitivo (**b**), Sugraone (**c**) and Negroamaro (**d**) plants treated with water (H_2O), *Trichoderma harzianum* T39 (T39) or benzothiadiazole (BTH). Relative expression levels (fold changes) were calculated using *Actin* as constitutive gene for normalization and data were calibrated on H_2O-treated Pinot noir plants before inoculation. F-test revealed non-significant treatment-experiment interactions ($P > 0.05$) and data from two repetitions of the experiment were pooled. Mean levels of relative expression and standard errors from six replicates (plants) pooled from the two experiments are presented for each treatment. Different letters indicate significant differences among treatments and cultivars, according to Fisher's test ($P < 0.05$) on fold change values transformed according to the equation $Y = Log_{10} (1 + x)$ (Casagrande et al., 2011).

The expression of four defence-related genes (Table 2) was analysed in the selected cultivars before and 24 h after *P. viticola* inoculation. Gene expression profiles were consistent in two independent repetitions of the experiment (F-test, $P > 0.05$) and data were pooled. Analysis of sequences obtained from real-time PCR products confirmed that the target genes were correctly amplified in each cultivar, with the exception of *PR-4* in Sugraone. Alignments of primer sequences to the Pinot noir predicted genes suggested that all isoforms of each gene were amplified in real-time PCR.

PR-1 was induced by *P. viticola* inoculation in H_2O-treated Pinot noir plants (Fig. 2a). *PR-1* was directly induced by BTH treatment before inoculation, and its expression remained high also at 24 h after *P. viticola* inoculation in BTH-treated plants. *PR-1* was not induced in T39-treated plants, suggesting that T39-induced resistance could be mediated by SA-independent pathways (Perazzolli et al., 2011). In Primitivo, *PR-1* expression was induced by *P. viticola* in H_2O-treated plants and the *P. viticola*-dependent up-regulation of *PR-1* was inhibited in T39-treated plants (Fig. 2b). As in Pinot noir, BTH treatment directly induced *PR-1*, which was not further affected by *P. viticola* inoculation. No significant up-regulation of *PR-1* expression was observed in Sugraone (Fig. 2c) and Negroamaro (Fig. 2d) plants, with the exception of a *P. viticola*-dependent up-regulation in BTH-treated Sugraone. The level of *PR-1* expression in uninoculated H_2O-treated plants was 4-fold greater in Negroamaro than in Pinot noir, suggesting a higher level of basal resistance.

PR-2 expression was induced by *P. viticola* inoculation in H_2O-treated Pinot noir plants (Fig. 3a) and it was directly induced by BTH and T39 treatments before pathogen inoculation. *PR-2* expression was enhanced after *P. viticola* inoculation in T39-treated plants, and the level resulted 2.1-fold greater in T39-treated than in H_2O-treated plants at 24 h after pathogen inoculation (priming effect). No significant changes in *PR-2* expression were observed in H_2O- and BTH-treated Primitivo plants (Fig. 3b). The expression level of *PR-2* was directly induced by T39, and it was 3.0-fold greater in T39-treated than in H_2O-treated Primitivo plants at 24 h after *P. viticola* inoculation. *PR-2* was not affected by any of the treatments in Sugraone (Fig. 3c) and in Negroamaro (Fig. 3d) plants. The basal level of *PR-2* expression in uninoculated H_2O-treated plants was 4-fold lower in Sugraone than in the other cultivars.

PR-4 was induced more than 4-fold by *P. viticola* in H_2O-treated Pinot noir plants (Fig. 4a). No direct induction and no priming effect of *PR-4* were observed in BTH-treated plants. T39

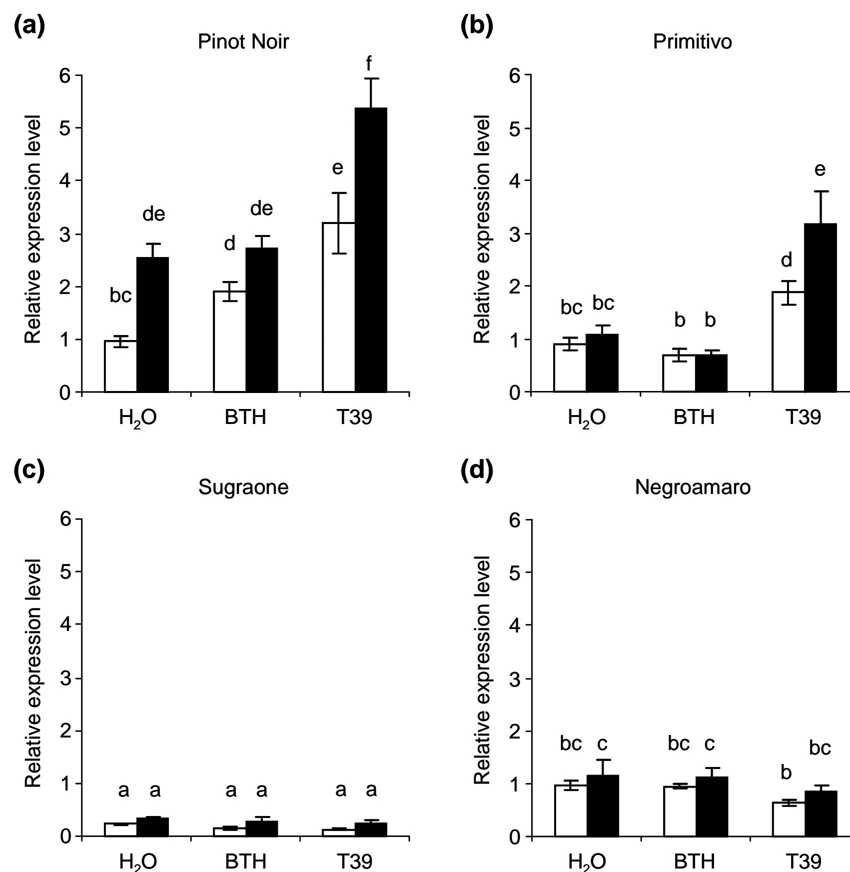

Figure 3. Expression of the defence gene encoding the pathogenesis-related protein 2 (*PR-2*) was analysed in leaf samples collected immediately before (white) and 24 h after (black) *Plasmopara viticola* inoculation of leaves of Pinot noir (**a**), Primitivo (**b**), Sugraone (**c**) and Negroamaro (**d**) plants treated with water (H_2O), *Trichoderma harzianum* T39 (T39) or Benzothiadiazole (BTH). Relative expression levels (fold changes) were calculated using *Actin* as constitutive gene for normalization and data were calibrated on H_2O-treated Pinot noir plants before inoculation. F-test revealed non-significant treatment-experiment interactions ($P > 0.05$) and data from two repetitions of the experiment were pooled. Mean levels of relative expression and standard errors from six replicates (plants) pooled from the two experiments are presented for each treatment. Different letters indicate significant differences among treatments and cultivars, according to Fisher's test ($P < 0.05$) on fold change values transformed according to the equation $Y = Log_{10} (1 + x)$ (Casagrande et al., 2011).

treatment caused direct induction (2.5-fold) and priming effect (1.8-fold) of *PR-4* expression, in agreement with our previous results (Perazzolli et al., 2011). The basal level of *PR-4* expression in uninoculated H_2O-treated plants was 6-fold lower in Primitivo (Fig. 4b) and 5000-fold lower in Negroamaro (Fig. 4c) than in Pinot noir plants. *PR-4* was induced by *P. viticola* in H_2O-treated Primitivo plants (inset of Fig. 4b). BTH treatment induced *PR-4* before pathogen inoculation and similar expression levels of *PR-4* were observed in inoculated BTH- and H_2O-treated Primitivo plants. T39 treatment caused direct induction and priming effect of *PR-4* in Primitivo plants. *PR-4* was induced by *P. viticola* inoculation in H_2O- and BTH-treated

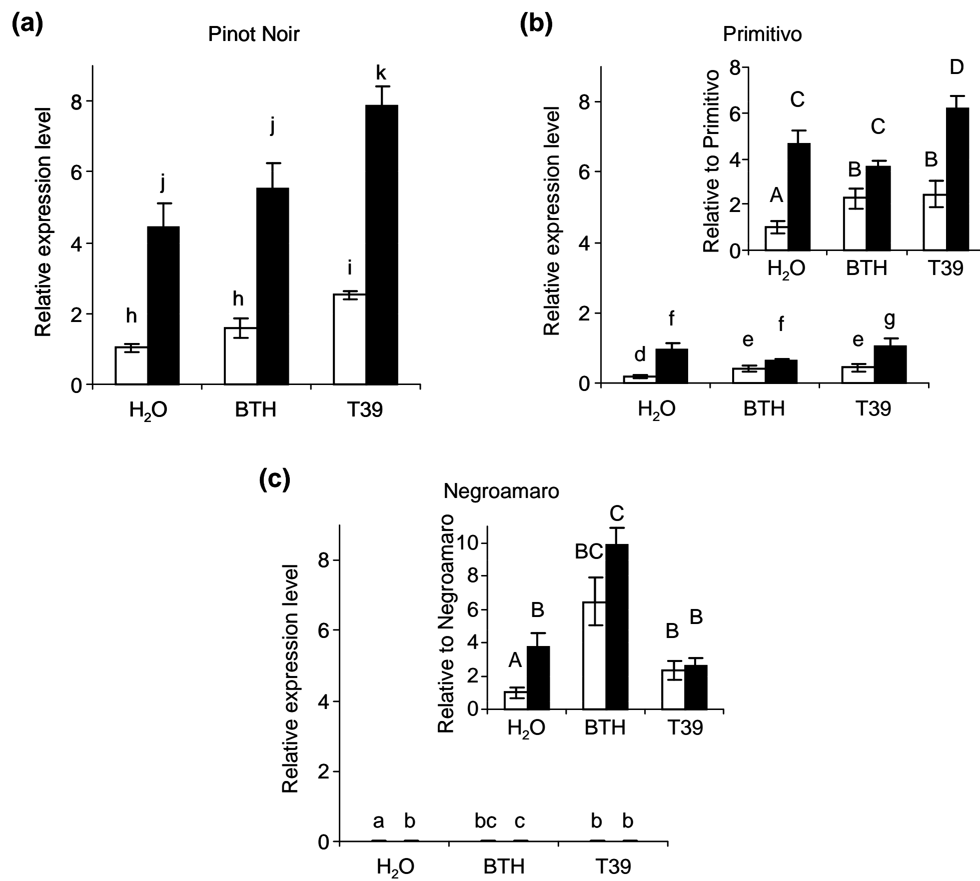

Figure 4. Expression of the defence gene encoding the pathogenesis-related protein 4 (*PR-4*) was analysed in leaf samples collected immediately before (white) and 24 h after (black) *Plasmopara viticola* inoculation of leaves of Pinot noir (**a**), Primitivo (**b**) and Negroamaro (**c**) plants treated with water (H_2O), *Trichoderma harzianum* T39 (T39) or Benzothiadiazole (BTH). Expression of *PR-4* was not detected in Sugraone. Relative expression levels (fold changes) were calculated using *Actin* as constitutive gene for normalization and data were calibrated on H_2O-treated Pinot noir plants before inoculation. F-test revealed non-significant treatment-experiment interactions ($P > 0.05$) and data from two repetitions of the experiment were pooled. Mean levels of relative expression and standard errors from six replicates (plants) pooled from the two experiments are presented for each treatment. Different lower-case letters indicate significant differences among treatments and cultivars, according to Fisher's test ($P < 0.05$) on fold change values transformed according to the equation $Y = Log_{10}(1 + x)$ (Casagrande et al., 2011). The insets represent fold changes calibrated on the expression in leaves of H_2O-treated Primitivo (**b**) and Negroamaro (**c**) plants before inoculation, respectively. For each inset, different upper-case letters indicate significant differences among treatments within each cultivar, according to Fisher's test ($P < 0.05$).

Negroamaro plants (inset of Fig. 4c). T39 treatment caused direct induction and no significant priming effect of *PR-4* in Negroamaro plants. *PR-4* expression was not detectable in Sugraone with the primer pairs designed on the Pinot noir sequence, probably due to the presence of polymorphisms in the gene sequences.

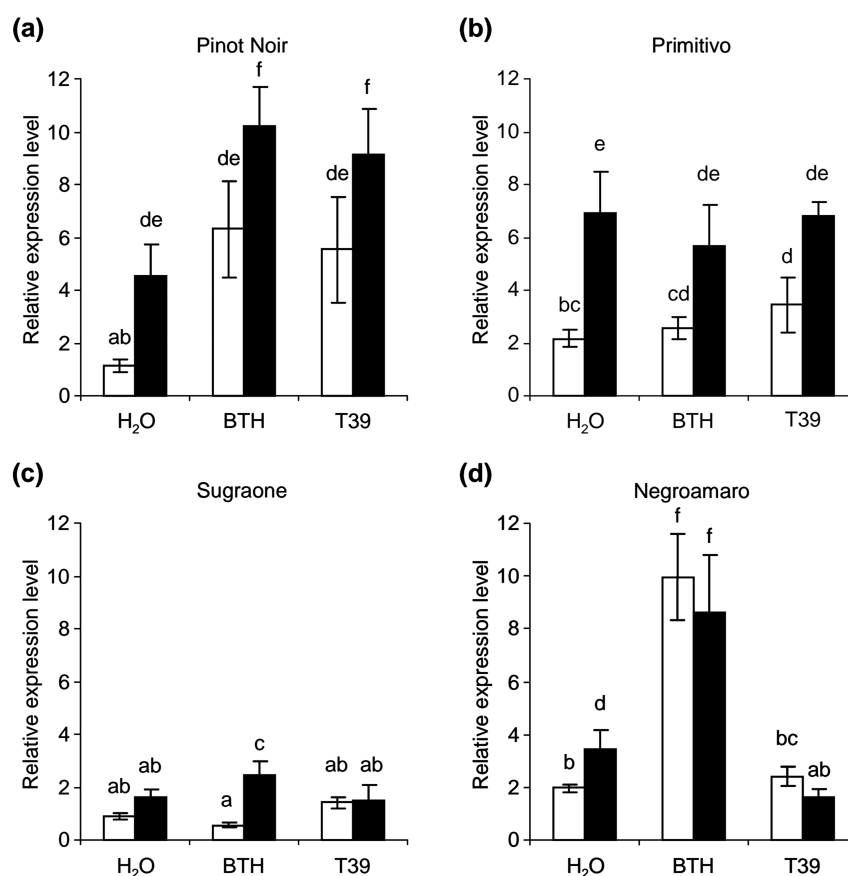

Figure 5. Expression of the defence gene encoding osmotin 1 (*OSM-1*) was analysed in leaf samples collected immediately before (white) and 24 h after (black) *Plasmopara viticola* inoculation of leaves of Pinot noir (**a**), Primitivo (**b**), Sugraone (**c**) and Negroamaro (**d**) plants treated with water (H_2O), *Trichoderma harzianum* T39 (T39) or Benzothiadiazole (BTH). Relative expression levels (fold changes) were calculated using *Actin* as constitutive gene for normalization and data were calibrated on H_2O-treated Pinot noir plants before inoculation. F-test revealed non-significant treatment-experiment interactions ($P > 0.05$) and data from two repetitions of the experiment were pooled. Mean levels of relative expression and standard errors from six replicates (plants) pooled from the two experiments are presented for each treatment. Different letters indicate significant differences among treatments and cultivars, according to Fisher's test ($P < 0.05$) on fold change values transformed according to the equation $Y = Log_{10} (1 + x)$ (Casagrande et al., 2011).

The osmotin 1 (*OSM-1*) gene, which belongs to the *PR-5* family, was induced more than 4-fold by *P. viticola* in H_2O-treated Pinot noir plants (Fig. 5a). T39 treatment caused direct induction of *OSM-1* before inoculation (5.5-fold) and priming effect (2.0-fold) for enhanced expression after pathogen inoculation compared with inoculated H_2O-treated plants. *OSM-1* expression was similar in BTH-treated and T39-treated Pinot noir plants, both before and after *P. viticola* inoculation. *OSM-1* was induced by *P. viticola* in H_2O-, BTH- and T39-treated Primitivo plants (Fig. 5b). Unlike Pinot noir, the expression levels of *OSM-1* were similar in T39-, BTH- and H_2O-treated Primitivo plants after pathogen inoculation. *OSM-1* was induced by *P. viticola*

in Sugraone plants treated with BTH, while no significant changes were observed with the other treatments (Fig. 5c). *OSM-1* was induced by *P. viticola* in H_2O-treated Negroamaro plants, and it was not affected by T39 treatment (Fig. 5d). *OSM-1* was directly induced (10-fold) by BTH treatment in Negroamaro plants and its expression was not further affected by *P. viticola* inoculation.

DISCUSSION

Induced resistance is a broad-spectrum disease control method based on the plant's own defences (Vallad and Goodman 2004; van der Ent et al., 2009) and is a promising low-impact approach for the control of crop diseases (Vallad and Goodman 2004). However, the full potential of induced resistance has yet to be realized mainly because of its inconsistency in field conditions (Walters et al., 2012; Walters et al., 2013), suggesting a lack of knowledge on the mechanisms underlying resistance activation in plants. Besides optimal timing of treatments and integration with other protection programmes (Walters et al., 2012), the efficacy of different resistance inducers and the influence of the plant genotype should be clarified for each crop (Walters et al., 2013) in order to maximize the effects of this biocontrol method.

The biocontrol agent T39 reduces downy mildew severity in the Pinot noir cultivar (Perazzolli et al., 2008; Perazzolli et al., 2011) by activating resistance through a complex reprogramming of the plant transcriptome (Perazzolli et al., 2012) and proteome (Palmieri et al., 2012). In this study, we have demonstrated that T39 is also effective in reducing the severity of downy mildew in other grapevine cultivars. However, different levels of T39 efficacy were observed, indicating a possible effect of the plant genotype in resistance activation. The highest levels of T39 efficacy were observed in the grapevine cultivars which were least susceptible to the disease (severity of disease in H_2O-treated plants), such as Negroamaro and Black magic. Conversely, T39-induced resistance was least effective in the Primitivo cultivar, which exhibited the highest level of disease severity in H_2O-treated plants. In contrast to T39, BTH activated a consistently high level of resistance with no differences among the grapevine cultivars. The different effects of T39 and BTH on grapevine cultivars could be therefore related to the type of resistance inducer and/or the defence signals elicited in the plant. A negative correlation between the susceptibility of control plants and the efficacy of T39-induced resistance was observed,

confirming that ISR is an enhancement of basal plant resistance (Pieterse et al., 2002). Plant genotype has been previously shown to influence the expression of induced resistance, and the relationship between basal and induced resistance seems to depend on the plant species and the resistance inducer. The highest level of induced resistance has been found in wild accessions of resistant bean (Córdova-Campos et al., 2012) and tobacco (Perez et al., 2003) and in partially resistant cucumber cultivars (Hijwegen and Verhaar 1995). On the other hand, resistance activation was found to be greater in highly susceptible than in partially resistant soybean (Dann et al., 1998) and tomato (Tucci et al., 2011) cultivars, but no relationship between levels of basal and induced resistance was observed in tomato (Sharma et al., 2010) and spring barley (Walters et al., 2011) genotypes.

In order to investigate the effect of the host genotype on T39-induced resistance, we analysed the transcriptional regulation of four defence marker genes in four grapevine cultivars. *PR-1* and *PR-4* genes were used as markers of SA- and JA-mediated responses, respectively (Hamiduzzaman et al., 2005). *PR-2* and *OSM-1* were used as markers of T39-induced resistance, on the basis of their direct induction and priming effect in T39-treated Pinot noir plants (Perazzolli et al., 2012; Perazzolli et al., 2011). Whereas the differences in defence gene expressions of resistant and susceptible *Vitis* genotypes have been widely investigated (Casagrande et al., 2011; Figueiredo et al., 2012; Polesani et al., 2010), in this study we showed that also different susceptible cultivars react differently to downy mildew inoculation. More specifically, *PR-1* was induced by *P. viticola* in H_2O-treated Pinot noir and Primitivo plants, while *PR-2* was induced only in Pinot noir plants. *PR-4* and *OSM-1* were induced by *P. viticola* in Pinot noir, Primitivo and Negroamaro. Moreover, the expression levels of *PR-2* and *PR-4* in inoculated H_2O-treated plants were greater in Pinot noir than in Primitivo, in agreement with the lower susceptibility to downy mildew.

Gene expression analysis showed that T39 has a dual effect in Pinot noir and Primitivo plants: it directly stimulates up-regulation of *PR-2* and *PR-4* and enhances expression of these genes after pathogen inoculation. Interestingly, the direct induction by T39 and the priming effect in T39-treated plants were less evident for *PR-2*, *PR-4* and *OSM-1* genes in Primitivo than in Pinot noir, in agreement with the lower efficacy of T39-induced resistance. However, different reactions to T39 were observed in the other two grapevine cultivars, Sugraone and Negroamaro, suggesting the involvement of more complex mechanisms or additional defence genes in induced

resistance processes. Changes in *PR* gene expression were less relevant in Sugraone and Negroamaro compared with Primitivo and Pinot noir during T39-induced resistance, indicating a natural variation in the reaction to the beneficial microorganism. In particular, T39-induced resistance was most effective in Negroamaro, which did not exhibit transcriptional changes of all tested genes in T39-treated plants. Moreover, the cultivars of table (Sugraone) and wine (Pino noir) grape showed comparable levels of T39 efficacy, but they differed significantly in the expression profiles of defence-related genes. In BTH-treated plants, the expression levels of *PR-1*, *PR-2* and *PR-4* were lower in Negroamaro and Sugraone than in Pinot noir, confirming that there are few transcriptional changes of *PR* genes in these cultivars. *OSM-1* was strongly induced by BTH in Negroamaro and Pinot noir plants, suggesting that this gene plays a role in BTH-activated defence in both cultivars. These findings suggested that the mechanisms of induced resistance are affected by genetic factors of different grapevine cultivars. However, we found no absolute correlation between the expression of defence genes and the efficacy of induced resistance in grapevine. Similar results have been previously reported in other pathosystems, and no positive correlations between the efficacy of *Trichoderma* spp.-induced resistance and the expression of *PR* genes were observed in tomato cultivars (Tucci et al., 2011). Likewise, no direct correlations between the efficacy of BTH and the activity of a defence-related enzyme were found in spring barley (Walters et al., 2011).

In conclusion, we showed that induction of grapevine resistance can significantly reduce the severity of downy mildew symptoms in wine and table grape cultivars under controlled greenhouse conditions. The efficacy of T39-induced resistance differed significantly among the grapevine cultivars, suggesting the importance of genetic background in ensuring optimal results of this biocontrol method. Grapevine cultivars exhibited different molecular reactions to the pathogen and to the resistance inducers, suggesting that differences in the receptors or in some cellular components of the signalling network might influence the plant response. In general terms, we did not see any absolute relationship between the efficacy of BTH or T39 and the expression levels of defence-related genes in grapevine. While *PR-1*, *PR-2*, *PR-4* and *OSM-1* are involved in resistance mechanisms, their expression profiles do not generally predict the efficacy of induced resistance. Analysis of *PR* gene expression partially helped in understanding the molecular processes that underline the genetic control of the interactions between beneficial and pathogenic microorganisms in different grapevine cultivars. As observed in tomato lines (Tucci

et al., 2011), the specificities of *PR* expression profiles revealed complex regulation of induced resistance mechanisms in the different varieties. Resistance is probably based on the activation of multiple genotype-dependent mechanisms in plants, and detailed knowledge of each pathosystem and each resistance inducer is needed for each crop in order to select the most responsive cultivars. Further studies are required to identify the major genetic determinants of *Trichoderma* spp. recognition and resistance induction in order to understand the cellular processes responsible for different reactions to the same stimulus. In practical terms, we showed that resistance inducers, such as T39, should preferably be applied to highly responsive cultivars in order to maximize resistance activation. However, further studies are required to implement these findings in plant protection strategies under field conditions. There is also a need for new natural resistance inducers, which can activate higher levels of resistance in less responsive cultivars.

ACKNOWLEDGEMENTS

The authors dedicate this article to the memory of our colleague Vito Simeone. This research was supported by the EU project *CO-FREE* (theme KBBE.2011.1.2-06, grant agreement number 289497) and the *Envirochange* project funded by the Autonomous Province of Trento. Authors' contributions: HB carried out the real-time RT-PCR experiments and co-wrote the paper; BR helped with the real-time RT-PCR, nucleotide sequences analysis and co-wrote the paper; BE carried out the greenhouse experiments; OG helped in the greenhouse experiments; CG supervised the experiments and revised the paper; IP and MP conceived the study, designed the experiments and coordinated all research activities.

REFERENCES

Allègre, M., Héloir, M. C., Trouvelot, S., Daire, X., Pugin, A., Wendehenne, D. and Adrian, M. (2009). Are grapevine stomata involved in the elicitor-induced protection against downy mildew? Mol Plant Microbe Interact. 8: 977-986

Aziz, A., Poinssot, B., Daire, X., Adrian, M., Bezier, A., Lambert, B., Joubert, J. M. and Pugin, A. (2003). Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopara viticola*. Mol Plant Microbe Interact. 12: 1118-1128

Aziz, A., Trotel-Aziz, P., Dhuicq, L., Jeandet, P., Couderchet, M. and Vernet, G. (2006). Chitosan oligomers and copper sulfate induce grapevine defense reactions and resistance to gray mold and downy mildew. Phytopathology. 11: 1188-1194

Boubakri, H., Wahab, M. A., Chong, J., Bertsch, C., Mliki, A. and Soustre-Gacougnolle, I. (2012). Thiamine induced resistance to *Plasmopara viticola* in grapevine and elicited host-defense responses, including HR like-cell death. Plant Physiol. Biochem. 120-133

Casagrande, K., Falginella, L., Castellarin, S. D., Testolin, R. and Di Gaspero, G. (2011). Defence responses in Rpv3-dependent resistance to grapevine downy mildew. Planta. 6: 1097-1109

Conrath, U., Beckers, G. J. M., Flors, V., Garcia-Agustin, P., Jakab, G., Mauch, F., Newman, M. A., Pieterse, C. M. J., Poinssot, B., Pozo, M. J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L. and Mauch-Mani, B. (2006). Priming: getting ready for battle. Mol Plant Microbe Interact. 10: 1062-71

Córdova-Campos, O., Adame-Álvarez, R. M., Acosta-Gallegos, J. A. and Heil, M. (2012). Domestication affected the basal and induced disease resistance in common bean (*Phaseolus vulgaris*). Eur. J. Plant Pathol. 2: 367-379

Dann, E., Diers, B., Byrum, J. and Hammerschmidt, R. (1998). Effect of treating soybean with 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) on seed yields and the level of disease caused by Sclerotinia sclerotiorum in field and greenhouse studies. Eur. J. Plant Pathol. 3: 271-278

Dercks, W. and Creasy, L. L. (1989). Influence of fosetyl-Al on phytoalexin accumulation in the *Plasmopara viticola*-grapevine interaction. Physiol. Mol. Plant Pathol. 3: 203-213

Dufour, M. C., Lambert, C., Bouscaut, J., Mérillon, J. M. and Corio-Costet, M. F. (2012). Benzothiadiazole-primed defence responses and enhanced differential expression of defence genes in *Vitis vinifera* infected with biotrophic pathogens *Erysiphe necator* and *Plasmopara viticola*. Plant Pathol. 2: 370-382

EPPO (2001). European and Mediterranean Plant Protection Organization (EPPO). Guidelines for the efficacy evaluation of fungicides: *Plasmopara viticola*. EPPO Bull. 313-317

Figueiredo, A., Monteiro, F., Fortes, A. M., Bonow-Rex, M., Zyprian, E., Sousa, L. and Pais, M. S. (2012). Cultivar-specific kinetics of gene induction during downy mildew early infection in grapevine. Funct Integr Genomics. 2: 379-386

Gessler, C., Pertot, I. and Perazzolli, M. (2011). *Plasmopara viticola*, the causal agent of downy mildew of grapes. Phytopathol Mediterr. 3-44

Godard, S., Slacanin, I., Viret, O. and Gindro, K. (2009). Induction of defence mechanisms in grapevine leaves by emodin- and anthraquinone-rich plant extracts and their conferred resistance to downy mildew. Plant Physiol. Biochem. 9: 827-37

Hamiduzzaman, M. M., Jakab, G., Barnavon, L., Neuhaus, J. M. and Mauch-Mani, B. (2005). Beta-aminobutyric acid-induced resistance against downy mildew in grapevine acts

through the potentiation of callose formation and jasmonic acid signaling. Mol Plant Microbe Interact. 8: 819-29

Harm, A., Kassemeyer, H.-H., Seibicke, T. and Regner, F. (2011). Evaluation of chemical and natural resistance inducers against downy mildew (*Plasmopara viticola*) in grapevine. Am J Enol Vitic. 2: 184-192

Hijwegen, T. and Verhaar, M. A. (1995). Effects of cucumber genotype on the induction of resistance to powdery mildew, *Sphaerotheca fuliginea*, by 2, 6-dichloroisonicotinic acid. Plant Pathol. 4: 756-762

Longa, C. M. O., Savazzini, F., Tosi, S., Elad, Y. and Pertot, I. (2009). Evaluating the survival and environmental fate of the biocontrol agent *Trichoderma atroviride* SC1 in vineyards in northern Italy. J. Appl. Microbiol. 5: 1549-57

Palmieri, M. C., Perazzolli, M., Matafora, V., Moretto, M., Bachi, A. and Pertot, I. (2012). Proteomic analysis of grapevine resistance induced by *Trichoderma harzianum* T39 reveals specific defence pathways activated against downy mildew. J. Exp. Bot. 17: 6237–6251

Perazzolli, M., Dagostin, S., Ferrari, A., Elad, Y. and Pertot, I. (2008). Induction of systemic resistance against *Plasmopara viticola* in grapevine by *Trichoderma harzianum* T39 and benzothiadiazole. Biol. Control. 2: 228-234

Perazzolli, M., Moretto, M., Fontana, P., Ferrarini, A., Velasco, R., Moser, C., Delledonne, M. and Pertot, I. (2012). Downy mildew resistance induced by *Trichoderma harzianum* T39 in susceptible grapevines partially mimics transcriptional changes of resistant genotypes. BMC Genomics. 660

Perazzolli, M., Raoatti, B., Bozza, E. and Pertot, I. (2011). *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defence without costs for grapevine. Biol. Control. 74-82

Perez, L., Rodriguez, M. E., Rodriguez, F. and Roson, C. (2003). Efficacy of acibenzolar-S-methyl, an inducer of systemic acquired resistance against tobacco blue mould caused by *Peronospora hyoscyami* f. sp. tabacina. Crop Protect. 2: 405-413

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 9: e45

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S. and Van Wees, S. C. (2009). Networking by small-molecule hormones in plant immunity. Nat. Chem. Biol. 5: 308-316

Pieterse, C. M. J., van Wees, S. C. M., Ton, J., van Pelt, J. A. and van Loon, L. C. (2002). Signalling in rhizobacteria-Induced systemic resistance in *Arabidopsis thaliana*. Plant Biol. 5: 535-544

Pinto, K. M. S., do Nascimento, L. C., de Souza Gomes, E. C., da Silva, H. F. and Miranda, J. d. R. (2012). Efficiency of resistance elicitors in the management of grapevine downy mildew *Plasmopara viticola*: epidemiological, biochemical and economic aspects. Eur. J. Plant Pathol. 4: 745-754

Polesani, M., Bortesi, L., Ferrarini, A., Zamboni, A., Fasoli, M., Zadra, C., Lovato, A., Pezzotti, M., Delledonne, M. and Polverari, A. (2010). General and species-specific transcriptional responses to downy mildew infection in a susceptible (*Vitis vinifera*) and a resistant (*V. riparia*) grapevine species. BMC Genomics. 117

Reuveni, M., Zahavi, T. and Cohen, Y. (2001). Controlling downy mildew (*Plasmopara viticola*) in field-grown grapevine with β-aminobutyric acid (BABA). Phytoparasitica. 2: 125-133

Ruijter, J. M., Ramakers, C., Hoogaars, W. M., Karlen, Y., Bakker, O., van den Hoff, M. J. and Moorman, A. F. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 6: e45

Sharma, K., Butz, A. F. and Finckh, M. R. (2010). Effects of host and pathogen genotypes on inducibility of resistance in tomato (*Solanum lycopersicum*) to *Phytophthora infestans*. Plant Pathol. 6: 1062-1071

Steimetz, E., Trouvelot, S., Gindro, K., Bordier, A., Poinssot, B., Adrian, M. and Daire, X. (2012). Influence of leaf age on induced resistance in grapevine against *Plasmopara viticola*. Physiol. Mol. Plant Pathol. 0: 89-96

Thuerig, B., Slaughter, A., Marouf, E., Held, M., Mauch-Mani, B. and Tamm, L. (2011). Site-specific field resistance of grapevine to *Plasmopara viticola* correlates to altered gene expression and was not modulated by the application of organic amendments. Eur. J. Plant Pathol. 2: 255-265

Trouvelot, S., Varnier, A. L., Allègre, M., Mercier, L., Baillieul, F., Arnould, C., Gianinazzi-Pearson, V., Klarzynski, O., Joubert, J. M., Pugin, A. and Daire, X. (2008). A beta-1,3 glucan sulfate induces resistance in grapevine against *Plasmopara viticola* through priming of defense responses, including HR-like cell death. Mol Plant Microbe Interact. 2: 232-43

Tucci, M., Ruocco, M., De Masi, L., De Palma, M. and Lorito, M. (2011). The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. Mol. Plant Pathol. 4: 341-54

Vallad, G. E. and Goodman, R. M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. Crop Sci. 6: 1920-1934

van der Ent, S., Koornneef, A., Ton, J. and Pieterse, C. M. J. (2009) Induced resistance – orchestrating defence mechanisms through crosstalk and priming. In: Annual Plant Reviews Volume 34: Molecular Aspects of Plant Disease Resistance, Wiley-Blackwell, 334-370

Velasco, R., Zharkikh, A., Troggio, M., Cartwright, D. A., Cestaro, A., Pruss, D., Pindo, M., Fitzgerald, L. M., Vezzulli, S., Reid, J., Malacarne, G., Iliev, D., Coppola, G., Wardell, B., Micheletti, D., Macalma, T., Facci, M., Mitchell, J. T., Perazzolli, M., Eldredge, G., Gatto, P., Oyzerski, R., Moretto, M., Gutin, N., Stefanini, M., Chen, Y., Segala, C., Davenport, C., Dematte, L., Mraz, A., Battilana, J., Stormo, K., Costa, F., Tao, Q., Si-Ammour, A., Harkins, T., Lackey, A., Perbost, C., Taillon, B., Stella, A., Solovyev, V., Fawcett, J. A., Sterck, L., Vandepoele, K., Grando, S. M., Toppo, S., Moser, C., Lanchbury, J., Bogden, R., Skolnick, M., Sgaramella, V., Bhatnagar, S. K., Fontana, P., Gutin, A., van de Peer, Y., Salamini, F. and Viola, R. (2007). A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. PLoS ONE. 12: e1326

Verhagen, B. W., Glazebrook, J., Zhu, T., Chang, H. S., van Loon, L. C. and Pieterse, C. M. J. (2004). The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. Mol Plant Microbe Interact. 8: 895-908

Walters, D. R., Avrova, A., Bingham, I. J., Burnett, F. J., Fountaine, J., Havis, N. D., Hoad, S. P., Hughes, G., Looseley, M., Oxley, S. J. P., Renwick, A., Topp, C. F. E. and Newton, A. C. (2012). Control of foliar diseases in barley: towards an integrated approach. Eur. J. Plant Pathol. 1: 33-73

Walters, D. R., Havis, N. D., Paterson, L., Taylor, J. and Walsh, D. J. (2011). Cultivar effects on the expression of induced resistance in spring barley. Plant Dis. 5: 595-600

Walters, D. R., Ratsep, J. and Havis, N. D. (2013). Controlling crop diseases using induced resistance: challenges for the future. J. Exp. Bot. 5: 1263-1280

Chapter 5

General conclusions

Population genetics studies on downy mildew epidemics clarified the contribution of primary and secondary infections to an epidemic. In contrast to the existing belief that primary infections occur only at early disease stages and in limited scale, it was shown that oosporic infections play a main role at the initiation of the disease and occur throughout the epidemiological season, with most of the genotypes having a limited ability to spread asexually (Gobbin et al., 2005; Rumbou and Gessler 2004). Moreover, while the majority of genotypes fail to reproduce further after primary infections, few of them have been found to produce secondary lesions and to predominate in summer populations of the pathogen (Matasci et al., 2010; Rumbou and Gessler 2004). Little is known about the mechanisms of selection of the predominant genotypes. Differences in aggressiveness components have been shown to be relevant in the process of selection among co-inoculation of different *P. viticola* isolates (Corio-Costet et al., 2011), thus explaining the observed differences in frequencies of various genotypes in *P. viticola* population during the season. However, other mechanisms of selection involving direct competition between isolates have been also hypothesized (Young et al., 2009). Un-revealing the principles of selection between genotypes during an epidemic may help in counteract strategies used by a restricted class of genotypes to predominate among others. Thus, the prevention of the establishment of predominant genotypes could improve the actual measures used for the control of downy mildew of grapevine.

To address this topic, in chapter 2 we described the collection of *P. viticola* isolates from different climatic regions and we evaluated differences in latency at non-optimal conditions. No difference were observed among the collected isolates, and we could exclude a link between the origin of the isolate and an advantage to better grow at sub-optimal conditions. To evaluate whether factors other than aggressiveness components could be responsible for genotype selection, we evaluated polycyclic infections of *P. viticola* in a competitive environment. The quantification of the isolates co-inoculated in the host has been developed on a method based on competitive microsatellite PCR (Naef et al., 2006; Reininger et al., 2011) optimized for *P. viticola*. Competitive PCR is a precise quantification tool (Zentilin and Giacca 2007) and the natural length polymorphism of the SSR markers served as mutual competitors. The method allowed us to differentiate and quantify the biomasses of different *P. viticola* genotypes within the same plant tissue, with sufficient resolution to study their frequency at several consecutive infection events. The quantification method based on competitive microsatellite PCR is quite

novel, moreover, this is the first study in which an interpolation on the calibration curve was not necessary. From the analysis of the electropherograms we could observe that genotypes strongly competed when co-inoculated, whereas the tested genotypes had comparable aggressiveness components and fitness indexes under individual inoculations. The mechanisms by which different genotypes compete are not well understood, however, for other oomycetes (i.e. *Phytophthora infestans*) it has been hypothesized that some genotypes could induce production of defense proteins in the host plant earlier than others in a competitive situation (Young et al., 2009). Thus, genotypes able to overcome the earlier plant defense reaction would have a competitive advantage, which, coupled with subsequent inhibition of other genotypes, could explain the competitive selection (Young et al., 2009). The genetic variability of effectors across isolates has been demonstrated in various oomycete species (Halterman et al., 2010) and, in *P. infestans* isolates, the genetic variability at one effector locus has also been correlated with pathogen aggressiveness (Halterman et al., 2010). Thus, as hypothesized for *P. infestans* genotypes (Young et al., 2009), the more competitive *P. viticola* genotypes could have a more complex arsenal of effectors enabling it to suppress or overcome host resistance, while the less competitive genotypes are more vulnerable to the host defense mechanism triggered by the more competitive genotype. The results and interpretations presented in chapter 2 represent a valuable addition to current knowledge of the biology of this grapevine pathogen. Plant-pathogen interactions seem to be more complex than mere colonization of plant tissue: different *P. viticola* genotypes reacted differently when co-existing on a unique substrate and differences in the production of effector molecules could contribute to the establishment of dominant genotypes in the field over the course of an epidemic season of downy mildew.

In order to develop alternatives to chemical fungicides for the control of downy mildew of grapevine, the biocontrol agent *Trichoderma harzianum* T39 (T39) was shown to activate the induced systemic resistance (ISR) in *Vitis vinifera* cv. Pinot Noir, reducing disease symptoms through complex reprogramming of the plant transcriptome (Perazzolli et al., 2012) and proteome (Palmieri et al., 2012). The induced resistance is a plant-mediated mechanism, and its expression under field conditions is likely to be influenced by a number of factors, including environment, genotype and crop nutrition (Walters et al., 2013). Plants respond differently to multiple stresses than to individual stresses (Atkinson and Urwin 2012) and more information on the effect of abiotic stresses and plant genotype on biocontrol tools based on induced resistance

is needed (Walters et al., 2005). Concerns about the impact of abiotic stresses on agriculture have been raised in the last decade, especially in light of the predicted effects of climate changes (Lobell et al., 2011). Heat waves and drought may occur with increased frequency, threatening crop production and influencing interactions with both pathogenic (Eastburn et al., 2011; Pautasso et al., 2012; Yasuda et al., 2008) and beneficial (Compant et al., 2010) microorganisms. Whereas the effects of abiotic stresses on plant resistance to pathogens have been extensively studied (Atkinson and Urwin 2012), their effects on the induced resistance mechanism has received little attention (Baysal et al., 2007). Also the plant genotype has been previously shown to influence the expression of induced resistance, and the relationship within basal and induced resistance seems dependent on the plant species and the type of resistance inducer (Walters et al., 2013). Thus, in order to maximize the effects of this biocontrol method, we evaluated the effect of abiotic stresses and of the plant genotype on the T39-induced resistance. In chapter 3 we investigated the effect of exposing grapevines to a short period of heat and/or drought stress and, in chapter 4, we characterized the T39-induced resistance in different grapevine varieties, comparing with the resistance activated by benzothiadiazole-7-carbothioic acid S-methyl ester (BTH).

In chapter 3 I described that exposure to combined heat and drought stresses (HDS) significantly reduced the efficacy of T39-induced resistance to downy mildew. Leaf water potential values and expression analysis of heat-stress related genes confirmed that plants reacted to water deficit and to heat. Modulation of the grapevine ISR marker genes was analysed during the T39-induce resistance. The abiotic stress negatively affected the basal expression of some defence-related genes and attenuated gene expression also during the T39-induced resistance, thus highlighting the impact of abiotic stresses on the defense response also at the molecular level. The abiotic stress response in plants is mainly controlled by ABA (Cramer et al., 2011) and this hormone is also involved in regulating defense responses to biotic stresses together with the SA, JA and ET signaling pathways (Robert-Seilaniantz et al., 2011). Our results showed that abiotic stresses antagonized T39-induced resistance to downy mildew in grapevine, affecting the real efficacy of this biocontrol agent in reducing disease symptoms. T39-induced resistance is mediated by JA and ET signaling pathways (Korolev et al., 2008; Perazzolli et al., 2011) and ABA signaling antagonizes the JA- and ET-mediated defense response (Anderson et al., 2004; Atkinson and Urwin 2012). A crosstalk between these hormonal signalings during T39-induced

resistance have been previously suggested observing T39-induced resistance not efficiently activated in ABA-impaired mutants (Korolev et al., 2008). The negative effect of abiotic stresses on T39-induced resistance found in grapevine are thus in agreement with literature data. Therefore, the effects of abiotic stresses on induced resistance seems highly dependent on the resistance inducer and the signaling molecules involved, demonstrating the need for specific characterizations for each inducer and pathosystem.

In chapter 4 we described that T39-induced resistance showed different efficacy values among different cultivars. The varieties with lowest susceptibility to downy mildew in control plants showed also the highest efficacy values upon T39 treatment. The inverse correlation between susceptibility to the disease and efficacy of T39-induced resistance confirms that ISR is basically an enhancement of the basal plant resistance (Pieterse et al., 2000). Conversely, BTH-activated resistance was highly effective against downy mildew and not affected by grapevine genotype. Thus, the effect of the different plant genotype on the induced resistance could be also related to the type of resistance inducer and/or defence signals elicited into the plant. Expression analysis of four defence-related genes was carried out on a highly (Negroamaro), weakly (Primitivo) and normally (Sugraone) responsive cultivar to the T39-induced resistance and compared to the reference cultivar Pinot Noir. The molecular mechanisms activated upon resistance inductions and pathogen inoculation were complex and effected by the plant genotype. In Primitivo and Pinot Noir plants, T39-induced resistance was based on a dual effect: direct modulation of defence-related genes and enhanced expression after pathogen inoculation. A correlation between gene expression level and T39 efficacy was observed in Primitivo and Pinot Noir plants, but different genes or more complex mechanisms were probably responsible for downy mildew resistance in Sugraone and Negroamaro. The reactions to the same resistance inducer differed among grapevine genotypes, indicating that specific receptors are probably involved in regulating plant responses.

The induction of resistance in grapevine is a reliable biocontrol method to reduce the severity of downy mildew symptoms. However, the efficacy of T39-induced resistance is strongly affected by abiotic stresses and by the plant genotype, indicating the relevance of the correct environmental condition and genetic background to optimize efficacy results in controlling the disease. Warmer and drier summers are predicted in several regions due to climatic changes (Lobell et al., 2011). Accurate studies investigating the thresholds of abiotic

stresses (alone or in combination) which affect crop responses to beneficial and pathogenic microorganisms need to be carried out, and the use of specific ISR marker genes will contribute to the quantitative analysis of the impact of abiotic conditions on induced resistance. The use of a responsive variety is also particularly important to optimize the efficacy of this biocontrol agent against downy mildew (chapter 4) and new natural resistance inducers able to activate high levels of resistance in the less responsive cultivars should be also explored.

REFERENCES

Anderson, J. P., Badruzsaufari, E., Schenk, P. M., Manners, J. M., Desmond, O. J., Ehlert, C., Maclean, D. J., Ebert, P. R. and Kazan, K. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. Plant Cell. 12: 3460-3479

Atkinson, N. J. and Urwin, P. E. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. J. Exp. Bot. 10: 3523-3543

Baysal, Ö., Gürsoy, Y. Z., Örnek, H., Çetinel, B. and Teixeira da Silva, J. A. (2007). Enhanced systemic resistance to bacterial speck disease caused by *Pseudomonas syringae* pv. tomato by dl-β-aminobutyric acid under salt stress. Physiol. Plant. 3: 493-506

Compant, S., Van Der Heijden, M. G. A. and Sessitsch, A. (2010). Climate change effects on beneficial plant–microorganism interactions. FEMS Microbiol. Ecol. 2: 197-214

Corio-Costet, M.-F., Dufour, M.-C., Cigna, J., Abadie, P. and Chen, W.-J. (2011). Diversity and fitness of *Plasmopara viticola* isolates resistant to QoI fungicides. Eur. J. Plant Pathol. 2: 315-329

Cramer, G., Urano, K., Delrot, S., Pezzotti, M. and Shinozaki, K. (2011). Effects of abiotic stress on plants: a systems biology perspective. BMC Plant Biol. 1: 163

Eastburn, D. M., McElrone, A. J. and Bilgin, D. D. (2011). Influence of atmospheric and climatic change on plant–pathogen interactions. Plant Pathol. 1: 54-69

Gobbin, D., Jermini, M., Loskill, B., Pertot, I., Raynal, M. and Gessler, C. (2005). Importance of secondary inoculum of *Plasmopara viticola* to epidemics of grapevine downy mildew. Plant Pathol. 4: 522-534

Halterman, D. A., Chen, Y., Sopee, J., Berduo-Sandoval, J. and Sànchez-Pérez, A. (2010). Competition between *Phytophthora infestans* effectors leads to increased aggressiveness on plants containing broad-spectrum late blight resistance. PLoS ONE. 5: e10536

Korolev, N., Rav David, D. and Elad, Y. (2008). The role of phytohormones in basal resistance and *Trichoderma*-induced systemic resistance to *Botrytis cinerea* in *Arabidopsis thaliana*. BioControl. 4: 667-683

Lobell, D. B., Schlenker, W. and Costa-Roberts, J. (2011). Climate trends and global crop production since 1980. Science. 6042: 616-620

Matasci, C., Jermini, M., Gobbin, D. and Gessler, C. (2010). Microsatellite based population structure of *Plasmopara viticola* at single vine scale. Eur. J. Plant Pathol. 4: 501-508

Naef, A., Senatore, M. and Défago, G. (2006). A microsatellite based method for quantification of fungi in decomposing plant material elucidates the role of *Fusarium graminearum* DON production in the saprophytic competition with *Trichoderma atroviride* in maize tissue microcosms. FEMS Microbiol. Ecol. 2: 211-220

Palmieri, M. C., Perazzolli, M., Matafora, V., Moretto, M., Bachi, A. and Pertot, I. (2012). Proteomic analysis of grapevine resistance induced by *Trichoderma harzianum* T39 reveals specific defence pathways activated against downy mildew. J. Exp. Bot. 17: 6237-6251

Pautasso, M., Döring, T., Garbelotto, M., Pellis, L. and Jeger, M. (2012). Impacts of climate change on plant diseases—opinions and trends. Eur. J. Plant Pathol. 1: 295-313

Perazzolli, M., Moretto, M., Fontana, P., Ferrarini, A., Velasco, R., Moser, C., Delledonne, M. and Pertot, I. (2012). Downy mildew resistance induced by *Trichoderma harzianum* T39

in susceptible grapevines partially mimics transcriptional changes of resistant genotypes. BMC Genomics. 1: 660

Perazzolli, M., Roatti, B., Bozza, E. and Pertot, I. (2011). *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defense without costs for grapevine. Biol. Control. 1: 74-82

Pieterse, C. M. J., Van Pelt, J. A., Ton, J., Parchmann, S., Mueller, M. J., Buchala, A. J., Métraux, J.-P. and Van Loon, L. C. (2000). Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. Physiol. Mol. Plant Pathol. 3: 123-134

Reininger, V., Grünig, C. and Sieber, T. (2011). Microsatellite-based quantification method to estimate biomass of *Endophytic phialocephala* species in strain mixtures. Microb. Ecol. 3: 676-683

Robert-Seilaniantz, A., Grant, M. and Jones, J. D. G. (2011). Hormone crosstalk in plant disease and defense: more than just JASMONATE-SALICYLATE antagonism. Annu. Rev. Phytopathol. 1: 317-343

Rumbou, A. and Gessler, C. (2004). Genetic dissection of *Plasmopara viticola* population from a Greek vineyard in two consecutive years. Eur. J. Plant Pathol. 4: 379-392

Walters, D., Walsh, D., Newton, A. and Lyon, G. (2005). Induced resistance for plant disease control: maximizing the efficacy of resistance elicitors. Phytopathology. 12: 1368-1373

Walters, D. R., Ratsep, J. and Havis, N. D. (2013). Controlling crop diseases using induced resistance: challenges for the future. J. Exp. Bot.

Yasuda, M., Ishikawa, A., Jikumaru, Y., Seki, M., Umezawa, T., Asami, T., Maruyama-Nakashita, A., Kudo, T., Shinozaki, K., Yoshida, S. and Nakashita, H. (2008). Antagonistic interaction between systemic acquired resistance and the abscisic acid–mediated abiotic stress response in *Arabidopsis*. The Plant Cell. 6: 1678-1692

Young, G. K., Cooke, L. R., Kirk, W. W., Tumbalam, P., Perez, F. M. and Deahl, K. L. (2009). Influence of competition and host plant resistance on selection in *Phytophthora infestans* populations in Michigan, USA and in Northern Ireland. Plant Pathol. 4: 703-714

Zentilin, L. and Giacca, M. (2007). Competitive PCR for precise nucleic acid quantification. Nat. Protocols. 9: 2092-2104

AKNOWLEDGEMENTS

This work wouldn’t be presented without the support of my family. Thanks for being there and loving me always.

I am grateful to all the Plant Pathology Group and the working group in FEM. I will remember you forever! Thank you for believing in me.

Special thanks goes to….

Paola for all the moments we shared in these years. You are a very special friend, I have been lucky to met you in this world! We’ll be apart soon but I hope we’ll meet again sometimes and do the things we like to do together! I wish you the best of luck and happiness, wherever you’ll go!

Michele for your constant support in the work during these years. You growth me up, while I was making mistakes (but also something right once in a while!)

Cristina for your wide knowledge, wisdom and your smiling patience.

Paolo for your faithful friendship. We met again by chance in Zurich, while we were walking through our lives.

To the awesome colleagues that shared the office with me in FEM in these last months, especially Monica, Luca, Livio, Luisa. Thank you for the enjoyable atmosphere you all contributed to create!

To Massimo for the numerous books you suggested me to read and for the many lunch break passed together, chatting about anything but work.

To the Garda Lake for giving moments of happiness and pure fun while riding fast on the water with a board and a sail. Thank you, Simone, for bringing me with you in these audacious and windy moments.

PUBLICATIONS

Published Works in peer reviwed journals:

Perazzolli, M., **Roatti, B**., Bozza, E., Pertot, I (2011). *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defense without costs for grapevine. Biological Control. 58:74-82.

Roatti, B., Gessler,. C., Perazzolli, M., Pertot, I (2013). Co-inoculated *Plasmopara viticola* genotypes compete for the infection of the host independently from the aggressiveness components. European Journal of Plant Pathology, Volume 136, Issue 2, pp 363-371.

Banani, H., **Roatti, B.**, Ezzahi, B., Gessler, C., Pertot, I., Perazzolli, M. Characterization of resistance mechanisms activated by *Trichoderma harzianum* T39 and benzothiadiazole to downy mildew in different grapevine cultivars. Plant Pathology, DOI: 10.1111/ppa.12089.

Roatti, B., Perazzolli, M., Gessler, C., Pertot, I. Abiotic stresses affect *Trichoderma harzianum* T39-induced resistance to downy mildew in grapevine. Phytopathology, DOI: http://dx.doi.org/10.1094/PHYTO-02-13-0040-R.

CURRICULUM VITAE

Benedetta Roatti

Born January 7th, 1983, Citizen of Bologna, Italy

University education

Bachelor in Biotechnology September 2002-May 2006
Università di Bologna, grade 103

Master in Medical Biotechnology September 2006-October 2008
Università di Firenze, grade 110
Magna Cum Laude

PhD Doctor of Science September 2009-April 2013
ETH Zürich,
Plant Pathology Group: Institute of Integrative Biology
In collaboration with: Fondazione Edmund Mach (FEM), Italy