Plasmopara viticola, the downy mildew of grapevine: phenotypic and molecular characterization of single sporangium strains infecting hosts with different resistance levels

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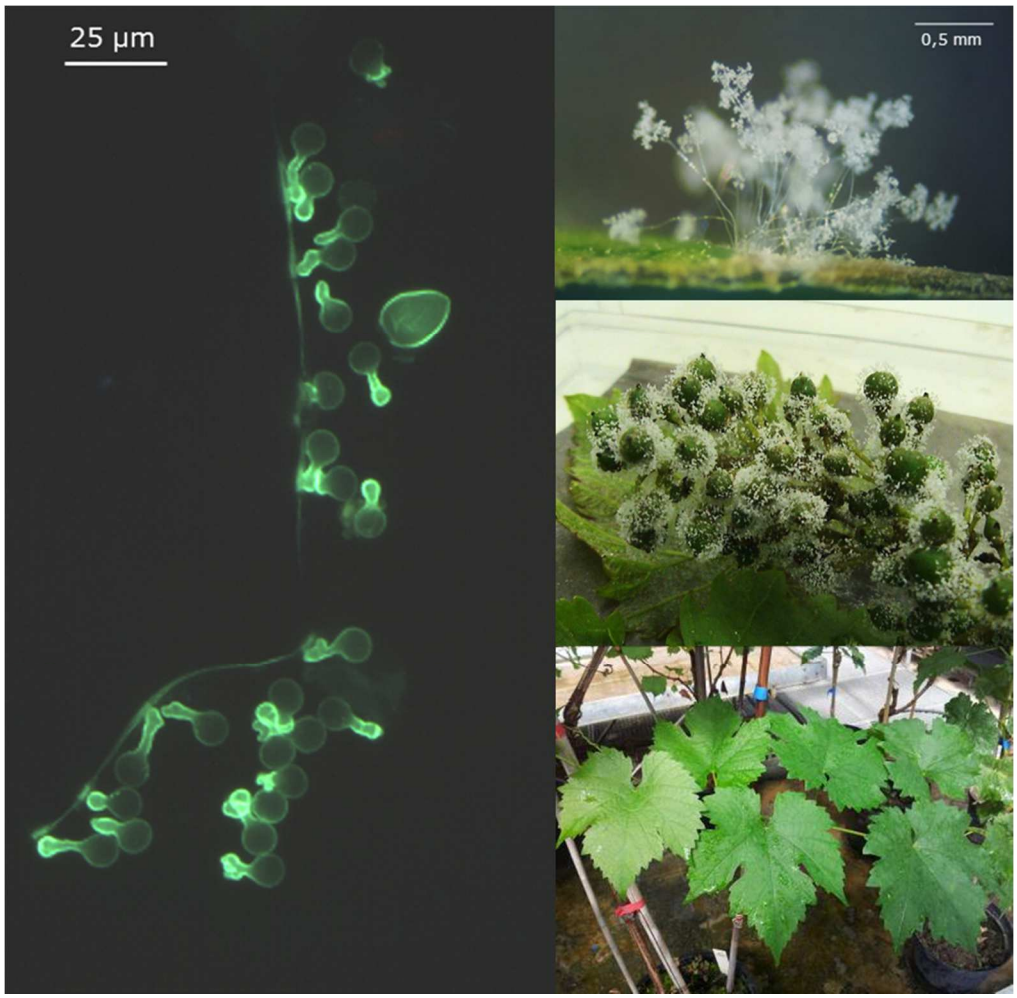

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To my dear parents, Eduardo and María Elena, who have always supported and encouraged me

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Preface

Plasmopara viticola, the downy mildew of grapevine (*Vitis vinifera*), is a very destructive pathogen involved in big losses on viticulture (Gessler *et al*., 2011). This oomycete is able to completely destroy a plantation if no measures are taken to control it. The main strategies to restrict this pathogen are forecast systems that can, by means of meteorological data, predict the time in which the pathogen may strike. Supported by this scheme, wine growers can apply fungicides in a more efficient way. However, still high amounts of fungicide have to be applied many times through the season to keep the pathogen under control (Gessler *et al*., 2011). A better understanding of the interaction between the plant and the pathogen is needed in order to develop new strategies to combat the oomycete.

The genetic variability of this destructive pathogen has not been sufficiently studied yet and no pathotypes or races have been identified like in other important oomycetes (Tourvieille De Labrouhe *et al*., 2000, Lebeda & Petrželová 2004, Petrželová & Lebeda 2010). A major constraint in this field is the biotrophic nature of the pathogen, which can only be cultivated on grapevine leaves. The perennial nature of *V. vinifera* supposes an even higher challenge when compared to annual hosts such as lettuce or sunflower which can easily be cultivated throughout the year when plant material is required for cultivation or testing. A leaf disc bioassay was established in chapter 1 to study the interactions between *P. viticola* and its host in the laboratory. North American wild species and representative *V*. *vinifera* cultivars were used to characterize more than 30 downy mildew isolates or strains. This technique provided a simple methodology to evaluate the capacity of specific strains to infect hosts with different levels of resistance and to establish a classification system for the pathogen phenotypes based on their virulence.

In order to achieve a more detailed characterization of the strains, a broadening of the system was studied in chapter 2. Asiatic and North American wild *Vitis* species were tested for interesting reactions when infected with the pathogen. High phenotypic differences were found using this system between the selected strains. As a consequence of the diversity in field populations, the application of a single sporing technique to obtain genetic homogeneous material for subsequent experiments was unavoidable.

This conclusion was also supported by the results obtained in chapter 3 which was aimed to compare strains with different virulence at the molecular level. Selected strains were characterized using two types of molecular markers (SNPs and SSRs) previously published for *P. viticola* (Gobbin *et al*., 2003a, Delmotte *et al*., 2006, 2011, Matasci *et al*., 2010, Rouxel *et al*., 2012). This characterization intended to achieve an unequivocal identification of defined genotypes of the pathogen bearing interesting characteristics. A high genotypic diversity was confirmed by this means between isolates from different fields but even within single sporangium strains from a field isolate.

In chapter 4, an important method for a better understanding of the infection mechanisms was designed, combining hosts which differ in susceptibility, with *P. viticola* strains of different virulence. Fluorescence microscopy enabled us to trace the development of the pathogen inside the tissue, even before symptoms became visible on the leaves. The growth of a highly virulent strain inside a tolerant host was observed and analyzed, in comparison with that of a lowly virulent strain.

Taking advantage of specific host-pathogen combinations, the expression of putative effector genes was studied in this chapter, intending to elucidate the mechanisms behind the difference in virulence observed. Special attention was given to oomycetes effectors in previous years to reveal how these group of pathogens are able to infect the plant, overcoming its immune reaction (Kamoun 2006, Hogenhout *et al*., 2009, Schornack *et al*., 2009, de Jonge *et al*., 2011, Bozkurt *et al*., 2012, Fawke *et al*., 2015, Oliveira-Garcia & Valent 2015). A screening in this chapter of, at the date known effectors of *P. viticola,* revealed at least five effectors with an interesting expression pattern that could be associated with the infection development and the reaction of the plant. From the plant side an upregulation of a gene from the surveillance system in the tolerant cultivar (McHale *et al*., 2006) shed light on the mechanism behind resistance against the pathogen. The ability to answer in a rapid way might play a crucial role for those highly virulent strains and its importance is here discussed. The suitability of *in vitro* germinated spores to study the gene expression in a host-free system was also investigated and contrasted with previous studies (Riemann *et al*., 2002).

Partial aspects of this project were simultaneously investigated in the B.Sc theses of Melanie Fröhler, Nele Bendel and Markus Kaiser and in the M.Sc thesis of Sandra Becker, for which this work provided the material, methodological and theoretical background.

Some of the results obtained in those projects were fundamental part of this work and are indicated on each chapter.

Chapter 1

Assessment of phenotypic diversity of *Plasmopara viticola* on *Vitis* genotypes with different resistance

The results presented in this chapter were published in: Gómez-Zeledón, Reinhard Zipper and Otmar Spring, 2013. Assessment of phenotypic diversity of *Plasmopara viticola* on *Vitis* genotypes with different resistance. Crop Protection 54:221-228.

1.1 Introduction

Plasmopara viticola, the causal agent of grapevine downy mildew, has been involved in big yield losses since it was first introduced in Europe in the 1870s. It is one of the most important pathogens in viticulture, and therefore many studies have been conducted aiming on a better understanding of its behavior in the field and an effective disease management, as it has been reviewed by Gessler *et al*., (2011). Cultivated grapevine originating from *Vitis vinifera* is highly susceptible to *P. viticola* and therefore, for a long time, fungicide treatment was the only measure available to control the disease. Several fungicide applications are necessary every year and resistance has already been found to the most common groups of site specific fungicides currently applied against downy mildew, namely phenylamides (Staub & Sozzi, 1981, Bosshard & Schuepp 1983, Leroux & Clerjeau 1985) and carboxilic acid amides (CAA) (Gisi *et al*., 2007, Blum *et al*., 2010). Only in the past few decades, resistance breeding partially replaced the chemical plant protection against fungal diseases in grapevine. Particularly in wild *Vitis* species of North America (e.g. *V. riparia, V. rupestris*) and Asia (e.g. *V. amurensis*) high tolerance against downy mildew was found and used to improve the resistance in commercial grapevine. However, still the vast majority of vineyards consist of fully susceptible varieties and their replacement with cultivars of higher resistance is time consuming and expensive. A major obstacle for this process is the unavailability of fully resistant grapevine genotypes and the lack of knowledge on the resistance mechanisms. Several studies have tried to identify resistance genes on wild *Vitis* and close species like *Muscadinia rotundifolia* against the pathogen, but only quantitative trait loci (QTL) have been found. The physiological mechanisms responsible for the partial resistance associated with these genomic regions remains widely undisclosed (Kortekamp & Zyprian 2003, Merdinoglu *et al*., 2003, Welter *et al*., 2007, Blasi *et al*., 2011). Further attempts to

unravel such mechanisms will not only depend on genetically well-defined host plants, but also need to take into account the genetic background of the pathogen in specific compatible or incompatible combinations.

Molecular studies have shown a high genotypic diversity in European populations of *P. viticola* (Stark-Urnau *et al*., 2000, Gobbin *et al*., 2005, 2006, Scherer & Gisi 2006) and its relevance for the development of resistance and in the epidemics of this pathogen (Rumbou & Gessler 2007). In addition, some recent reports identified the possible occurrence of cryptic species in the *P. viticola* complex of North American accessions of the pathogen (Schröder *et al*., 2011, Rouxel *et al*., 2013). Nevertheless, there is still little information available about the phenotypic diversity of physiological isolates of *P. viticola* causing problems in viticulture. Some authors have tested the virulence of strains against *Vitis* spp. and cultivars and have found important differences, but races or pathotypes have not yet been characterized as in other downy mildews such as *Plasmopara halstedii* (Tourvieille De Labrouhe *et al*., 2000), *Pseudoperonospora cubensis* (Lebeda & Widrlechner 2004) or *Bremia lactucae* (Lebeda & Petrželová 2004, Petrželová & Lebeda 2010).

In addition, while the previous studies have mostly been based on genetically undefined and potentially inhomogeneous *P. viticola* samples subcultured from field isolates (Kiefer *et al*., 2002, Gindro *et al*., 2003, Kortekamp *et al*., 2003, Jürges *et al*., 2009, Unger *et al*., 2007, Alonso-Villaverde *et al*., 2011), other reports had found a high genetic diversity between such field samples of the pathogen (Kast 2001, Scherer & Gisi 2006). Molecular studies have shown that even within single field isolates, different genotypes of the pathogen can be present (Stark-Urnau *et al*., 2000, Gobbin *et al*., 2003a). Taking this into account, assessments of the phenotypic variation for defining pathotypes should start from genetically homogeneous strains. Attempts have been made to achieve this goal by using single sporangiophores for reinfection (Wong & Wilcox 2000, Wong *et al*., 2001, Gisi *et al*., 2007), but there is still no certainty that all sporangia in a sporangiophore are genetically identical or if they might vary due to an emergence from a heterokaryotic mycelium. Alternatively, monosporangia and monozoospore cultures have been used and proved to be stable in their sensitivity against a site specific fungicide during subculturing (Genet & Jaworska 2013).

A further prerequisite for pathotype differentiation should be the selection of standardized host genotypes (similar to the sunflower differentials with defined resistance genes for downy mildew). Despite studies on the mechanisms of resistance of wild *Vitis* species (Mysore & Ryu 2004) and the identification of different resistance reactions at various levels (Denzer *et al*., 1995a, Díez-Navajas *et al*., 2008, Jürges *et al*., 2009), the definition of suitable host genotypes is still a challenging task. QTLs associated with resistance to *P. viticola* have been reported for some grapevine cultivars (Merdinoglu *et al*., 2003, Fischer *et al*., 2004, Bellin *et al*., 2009, Blasi *et al*., 2011, Moreira *et al*., 2011) used in breeding programs, but not all of them are commonly available and many *Vitis vinifera* genotypes with an interesting observed resistance have not been characterized yet. We therefore compromised and decided to select a set of grapevine plants representing commercially used cultivars of *V. vinifera* with suggested resistance loci (*Rpv*) to *P. viticola* (VIVC 2015) and wild *Vitis* species which were expected to show a broad array of resistance reactions.

Finally, a suitable infection bioassay with defined resistance reactions was attempted for this study. A promising methodology is the leaf disc technique, which has been found efficient and practical for the evaluation of virulence and fungicide resistance of *P. viticola* (Denzer *et al*., 1995b, Wong & Wilcox 2000, Deglène-Benbrahim *et al*., 2010) and other oomycetes such as *Plasmopara halstedii* (Spring *et al*., 1997, Rozynek & Spring 2001, Spring & Zipper 2006), *Bremia lactucae* (Cohen *et al*., 2008) or *Peronospora tabacina* (Xie & Kúc 1997). A good correlation was found between this method and field studies (Brown *et al*., 1999), making it suitable for pathogen differentiation. A visual scale similar to that of OIV-452 (IPGRI 1997) has already been used to determine the degree of resistance of grapevine genotypes against downy mildew in leaf discs (Deglène-Benbrahim *et al*., 2010, Calonnec *et al*., 2012). However, the OIV scale was designed for the evaluation of whole leaves, after a long infection period and a specific application technique of the sporangia. For the expected amount of pathogen samples, the number of repetitions and the short period of infection in our assays, the proposed OIV system was not suitable. We therefore defined a new system to classify the isolates of *P. viticola* according to the intensity of sporulation and observed resistance reaction on specific host genotypes. The evaluation was based on five categories ranging from full susceptibility with profuse sporulation (type A) to complete resistance with no sporulation or necrotic reaction (type E).

We report here on the diversity of pathogen phenotypes between single sporangium strains of grapevine downy mildew collected from different fields and different host cultivars in South Germany and the adjacent region in France. This diversity was as assessed in a standardized leaf disc inoculation test with specific host genotypes.

1.2 Material and Methods

1.2.1 Cloning and propagation of the pathogen

Five field isolates of *P. viticola* (Berk. & Curt.) Berl. & de Toni collected from infected grapevine leaves of different wine growing regions - four in South Germany and one in France - were used in this study (Table 1.1). Due to their high susceptibility to the pathogen, leaves of *Vitis vinifera* L. cv. Müller-Thurgau and cv. Bacchus were used to propagate the oomycete in the laboratory. In order to assess the diversity within the field isolates, single sporangium strains (Table 1.1) were established using a method previously designed to achieve *P. halstedii* single sporangium infections (Spring *et al*., 1998). Young leaves (between the fourth and the seventh leaf from the shoot tip) were briefly rinsed with distilled water and 1 cm^2 leaf discs were cut out using a scalpel or a cork borer. Leaf discs were placed top-down in a 25 well plate (25 compartments square Petri dish; Sterilin® Ltd., Cambridge, United Kingdom) filled with 800 µL distilled water in each compartment. Sporangia were delivered on a Petri dish (60 x 15 mm) containing semisolid 1% water-agar medium and collected under an inverted microscope with a micromanipulator (Brinkmann Instrumentenbau, Mannheim, Germany) equipped with a glass capillary (Eppendorf transfer tips, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany, 80 µm diameter). Sporangia were placed individually on leaf discs with a drop of water and kept in a climate chamber at 18°C, the first 24 h in darkness and then under a 14 h photoperiod. The sporulation produced on every leaf disc was sub-cultured individually by submerging and shaking the disc in distilled water. The sporangia suspension was used as inoculum on which small young leaves were floated bottom-down in darkness at 18°C for 24 h. Subsequently, the water was discarded and the leaves were placed top-down on wet filter paper in closed plastic boxes for further incubation (18°C, 14 h photoperiod). Sporulation was found after 4-5 days and the process was repeated to recover more sporangia. Freshly developed sporangia were harvested in dry form by means of a suction device developed in our laboratory. They were either used directly for infection experiments or stored at -70 °C for later use.

Table 1.1: Field isolates and the single sporangium strains developed from it.

Code of field isolate	Collection date	Origin	Host cultivar	Coding for single sporangium strains
1117	7/2011	Colmar / F	Cabernet Sauvignon	A7, A17, A21, B6, B7, B12
1135	7/2011	Freiburg WBI / D	Müller-Thurgau	A21, F2, F6, F12, F15, H14
1136	7/2010	Pfaffenweiler / D	Regent	A15, A21, B6, B16
1137	7/2010	Pfaffenweiler/ D	Gutedel	A10, B3, C20, D13, D16, E19
1191	8/2011	Lauffen / D	Lemberger	A5, A7, B6, B8, B9, B11, B12, B18

1.2.2 Host genotype test

Cloned cuttings of six grapevine genotypes with different levels of resistance against downy mildew were generously provided by Prof. Dr. Hans-Heinz Kassemeyer, Institut of Enology and Viticulture in Freiburg (WBI), Prof. Dr. Peter Nick, Karlsruhe Institute of Technology and Prof. Dr. Ralf Vögele, Institute of Phytomedicine, University of Hohenheim. Original material of these genotypes is located in the collections of the WBI Freiburg (FR) or Karlsruhe Institute of Technology (Table 1.2). Plants were raised and kept at 25°C in a greenhouse of the Institute of Crop Science, University of Hohenheim with the kind assistance of Dr. Nikolaus Merkt. A system was employed using 1 cm^2 leaf discs in 25 well plates to characterize the virulence of the downy mildew isolates on each of the selected host genotypes. Following the same procedure described above, ten leaf discs of each genotype were placed top-down in the 25 well plates filled with 800 µl of distilled water. Sporangia suspensions were prepared for each *P. viticola* isolate or strain with distilled water. A drop of suspension (10-20 µl) with approximately 10 000 sporangia (scored in a Fuchs-Rosenthal counting chamber) was applied on the surface of each leaf disc. This high concentration of sporangia was chosen because the infectivity of sporangia can vary significantly from one experiment to the other. The experience showed that inoculations with less than 1000 sporangia delivered inconstant results. A drop of the sporangia suspension was placed in water to check the germination rate of the isolates after 24 h and thus evaluating the viability of the inoculum. The plates were incubated at 18°C, the first 24 h in darkness, and then under a photoperiod of 14 h. The inoculation drops were removed from the discs after two days to avoid developing of infections by molds or bacteria. After 10 days the infection rate of the strains on each host genotype was evaluated using the scale shown in Fig. 1.1. The scale was based on

the level of sporulation and the strength of the necrotic reaction present in the leaf discs, ranging from a profuse and not clearly restricted sporulation (A), to no reaction at all (E). This scale reflected the resistance of the host genotypes against each strain.

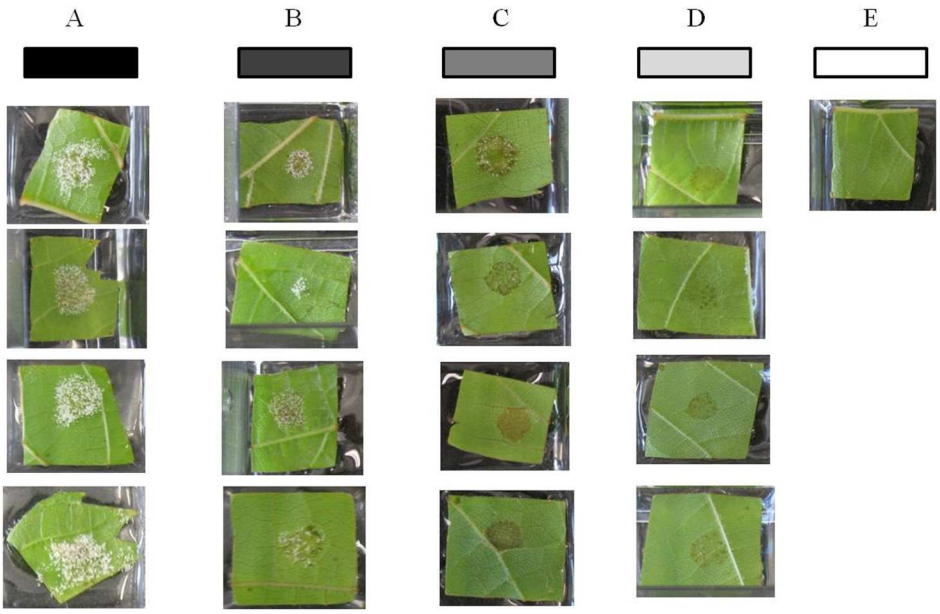

Figure 1.1: Host genotype reaction of *Vitis* in leaf disc inoculations with *Plasmopara viticola*. The categories differentiate between full susceptibility without necrotic reaction and unrestricted sporulation (A), moderate sporulation limited to the site of inoculation (B), strong necrotic reaction with scattered sporulation (C), light necrotic reaction with rare sporulation (D), and no necrotic reaction or sporulation (E).

In cases where the reaction type between categories was unclear (intermediate reaction between the ten discs, e.g. A and B or C and D) or when the infection ratio in the control (Müller-Thurgau) was below 80%, complete tests were repeated until the results were uniform and the infection on Müller-Thurgau was 100%. We did not observe shifts of more than one level within the ten discs of a sample and the categories assigned for host-pathogen combinations were stable from test to test (data not shown).

Table 1.2: Host genotypes used in the leaf disc bioassay.

Groups	Host genotypes
Group 1: *Vitis vinifera* cultivars	Müller-Thurgau (MT), ID: FR3 vg
	Regent (REG), ID: rpv.3
	Cabernet Cortis (CAB), ID: FR680
Group 2: wild *Vitis* species	*Vitis vinifera* L. ssp. s*ylvestris* (SYL), WBI
	Vitis rupestris (RUP), Vrup-01, ID: 5888
	Vitis riparia (RIP), Vrip-01, ID: 6548

1.2.3 Fungicide test

Two fungicides with different modes of action were used as described by Rozynek and Spring (2001) to evaluate the resistance reaction of the strains. The locally systemic fungicide dimethomorph (DIM) was tested in concentrations of 0,1 and 1 µg/ml and the systemic fungicide metalaxyl-M (MET) was tested in concentrations of 1 µg/ml and 10 µg/ml. In the same way as in the host genotype test, 800 µl solutions of the respective fungicide were placed in every compartment instead of water. The sporangia suspensions were prepared in every fungicide solution and adjusted using a Fuchs-Rosenthal counting chamber. A drop (10-20 µl) containing approximately 10 000 sporangia was placed on each 1 cm^2 leaf disc. Ten discs were used per fungicide concentration. Plates were incubated as described before and evaluated after 10 days using a scale of three categories: No resistance ▭; moderate resistance ▨ (0,1 µg/ml DIM or 1 µg/ml MET) and high resistance (1 µg/ml DIM or 10 µg/ml MET) ▩. According to the experience in our laboratory, sensitive strains were not able to sporulate at 0,1 µg/ml dimethomorph and 1 µg/ml metalaxyl-M. Hence moderate resistance was recorded when a strain sporulated on 0,1 µg/ml of dimethomorph or 1 µg/ml of metalaxyl-M and high resistance was assumed when sporulation occurred on 1 µg/ml of dimethomorph or 10 µg/ml of metalaxyl-M. In general, the tests were repeated at least twice, and all tests were repeated when the infection ratio in the control (Müller-Thurgau) was below 80%.

1.3 Results

Field isolates of *P. viticola* collected on different grapevine cultivars (Table 1.1) and from 3 distant geographical regions in South Germany (South-Baden, North-Württemberg) and France (Alsace) were cultivated under laboratory conditions on detached leaves of *Vitis vinifera* L. cv. Müller-Thurgau and Bacchus in order to screen their pathogenic phenotype and for the generation of single sporangium strains. Out of approximately 350 attempts, a total of 30 single sporangium strains could be developed and propagated in sufficient amounts for the characterization of pathogenic phenotypes. Although the use of frozen sporangia would be more practical, fresh sporangia (those collected from the infected leaves for immediate use) showed a much higher germination rate and gave more consistent results (data not shown). Freshly harvested sporangia were therefore used for the leaf disc assays.

1.3.1 Host resistance reactions

The leaf disc bioassays revealed five categories of infection/resistance ranging from full susceptibility (type A reaction) to the complete absence of symptoms (type E reaction, or so called nonhost resistance) (Fig. 1.1). In some cases, the boundaries between categories were fluent (e.g. type A/B or C/D) so that unique definition was difficult. In such cases the assignment was made according to the majority of reactions found in the ten discs per sample. We did not observe deviations of more than one level (e.g. A and C) between discs of one sample and the assigned categories remained stable in repeated tests. The intensity of sporulation present in the leaf discs was a good indicator for the virulence of the strain. Similarly, the degree of necrosis indicated the incompatibility of each host-pathogen combination.

1.3.2 Phenotypic variability between field isolates

Important differences were found between field isolates (Fig. 1.2). While all of them profusely sporulated on the leaves of the *Vitis* cultivar Müller-Thurgau (positive control), just two (1136 and 1137) produced strong sporulation in the tolerant cultivar Regent. These two field isolates were also able to infect the host genotypes in group 2 with mostly moderate sporulation, but differed in virulence towards *V. rupestris* (type A reaction of 1136) and *V. riparia* (strong necrotic reaction with 1137). Interestingly, the isolate 1135 from the same area in South-Baden showed the weakest virulence of all

field isolates and was unable to infect the cultivar Cabernet Cortis. In contrast, this cultivar showed higher susceptibility to the isolates 1117 (France) and 1191 (North-Württemberg), when compared to Regent. Within the wild host genotypes in group 2, *V. riparia* appeared to be the least susceptible. However, none of the tested host genotypes was completely resistant to all of the field isolates.

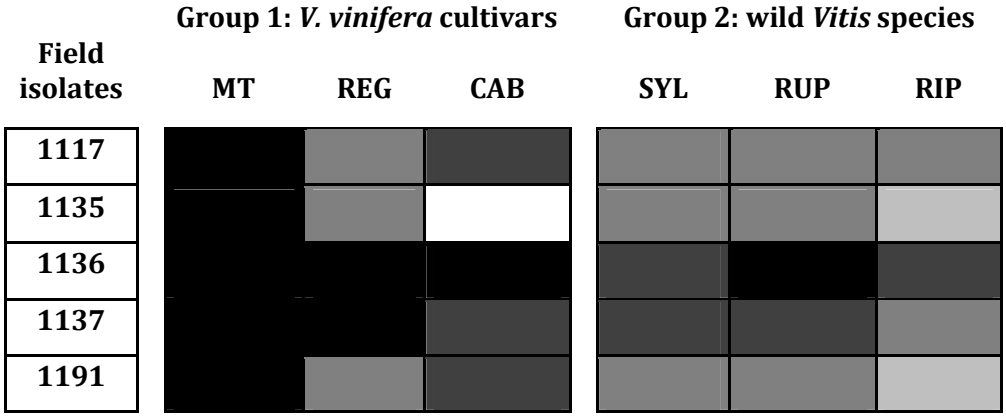

Figure 1.2: Genotype reaction test of the five field isolates of *P. viticola* on the six grapevine genotypes: **A** ■ Profuse sporulation; **B** ■ Moderate sporulation; **C** ■ Strong necrotic reaction with scattered sporulation; **D** ■ Rare sporulation with light necrotic reaction; **E** □ No sporulation or necrotic reaction.

1.3.3 Variability between single sporangium strains

The single sporangium strains presented very diverse reactions on the genotypes tested, not only when compared with strains from different fields, but also amongst sister strains from the same field isolate (Fig. 1.3). Only three combinations (1117-A21 and B6; 1135-F15 and H14; 1137-D13 and D16 and E19) showed identical reactions between strains from the same field isolate when the fungicide reaction (see below) was considered. Thus we found 25 different phenotypes out of 30 strains tested. Just one strain produced a moderate sporulation (1191-B15) on *V. riparia*, while others achieved just scattered or rare sporulation on this host. Only two strains did not infect at all. *Vitis rupestris* showed mostly moderate resistance presenting scattered sporulation together with a strong necrotic reaction. However, all four strains of field isolate 1136 gave type B reaction on this species (similar to one strain from each of isolates 1135, 1137 and 1191). *Vitis vinifera* L ssp. *sylvestris* also presented a moderate to low resistance, but this time more towards the strains of the field isolate 1137 (two of which produced profuse sporulation, similar to strain 1191-B9). Similarly, all isolates of 1137 showed a type A reaction on Regent, whereas Cabernet Cortis was able to restrict their sporulation almost entirely. In some cases, the reaction type of a strain was not found in the

respective field isolate and *vice versa*. This is probably due to the low ratio of this genotype in the field population and the random and limited selection of sporangia for cloning.

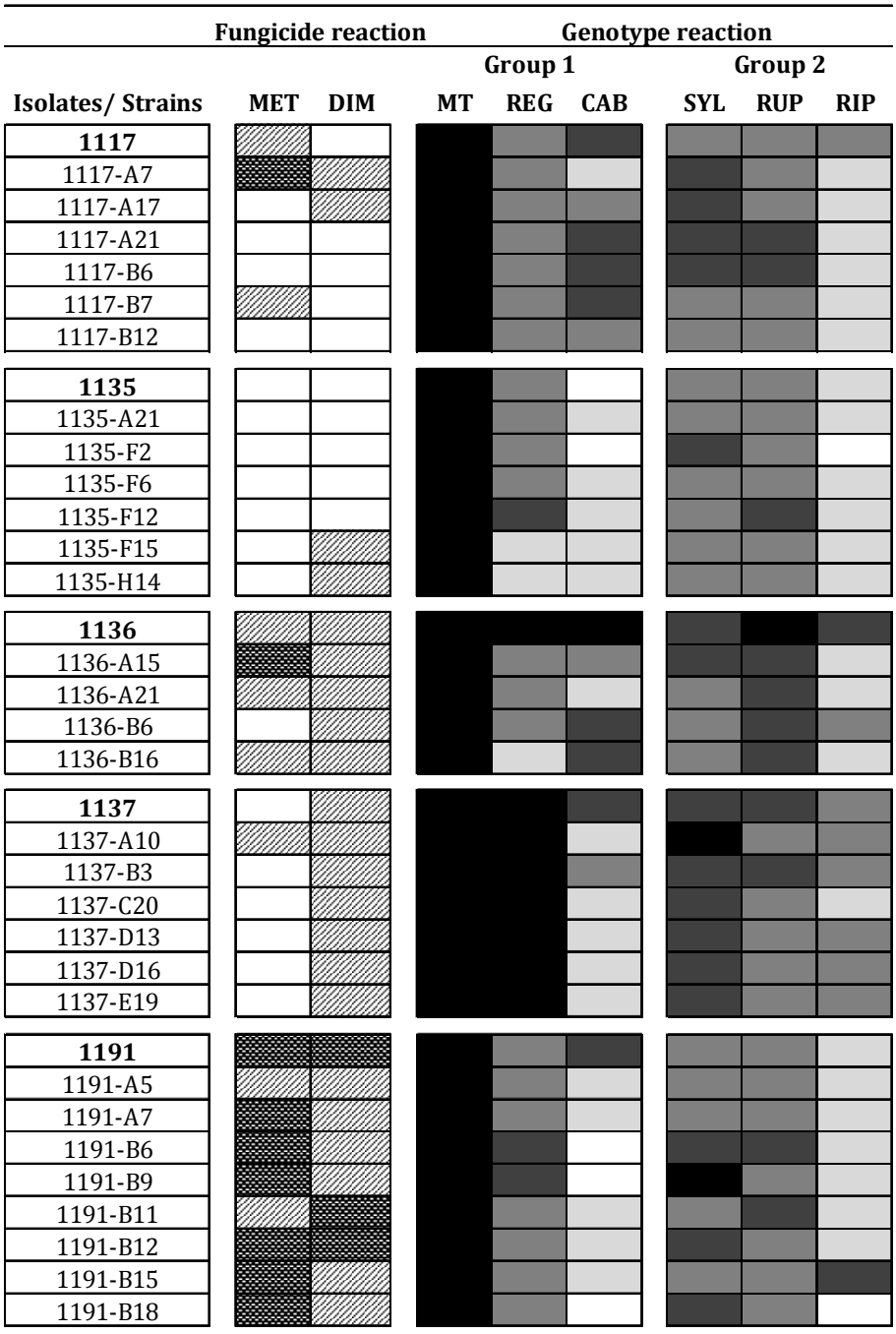

Figure 1.3: Fungicide and genotype reaction test. Metalaxyl (MET); dimethomorph (DIM); *V. vinifera* L. cv. Müller-Thurgau (MT); *V. vinifera* L. cv. Regent (REG); *V. vinifera* L. cv. Cabernet Cortis (CAB); *V. vinifera* L. ssp. *sylvestris* (SYL); *Vitis rupestris* S. (RUP); *Vitis riparia* Michx (RIP). *Fungicide reaction*: No resistance □; moderate resistance (0,1 µg/ml DIM or 1 µg/ml MET) ▨; high resistance (1 µg/ml DIM or 10 µg/ml MET) ▩. *Genotype reaction*: **A** ■ Profuse sporulation; **B** ■ Moderate sporulation; **C** ■ Strong necrotic reaction with scattered sporulation; **D** ■ Rare sporulation with light necrotic reaction; **E** □ No sporulation or necrotic reaction.

1.3.4 Fungicide test

The behavior of the isolates was very diverse in the fungicide test as well (Fig. 1.3). The strains of isolate 1135 were very sensitive to both fungicides tested, and just two strains exhibited moderate tolerance to dimethomorph (0,1 µg/ml). In contrast, all strains of isolate 1191 presented some degree of resistance. Some of them were even able to sporulate at 100 µg/ml of metalaxyl-M (toxic to the plant) and 5 µg/ml of dimethomorph (data not shown). In the field isolate 1137, all strains showed a moderate resistance against dimethomorph (0,1 µg/ml) while just one of them resulted moderate resistant to metalaxyl-M (1 µg/ml). It was interesting to find that within the strains of isolates 1117 and 1136, there were two with a high level of resistance to metalaxyl-M (10 µg/ml) and others with no resistance at all, showing that the diversity between sporangia of a field isolate was high.

1.4 Discussion

1.4.1 Pathogen management and reliability of infection studies

Resistance breeding and fungicide treatment have been fundamental measures used to control downy mildew in grapevine for a long time. However, the high diversity of the pathogen observed in virulence tests on *Vitis spp.* or cultivars (Li *et al*., 1986, Kast 1996, 2001, Jürges *et al*., 2009, Peressotti *et al*., 2010) and supported by results of molecular genetic studies (Gobbin *et al*., 2005, 2006), accounts for the limited success. Nevertheless, there appears no alternative other than the exploration of new sources of resistance for the protection of vineyards against *P. viticola,* even if it may only lead to temporary success. A system to differentiate virulent phenotypes in the population of the pathogen would be helpful and desirable, but unlike as in case of other economically relevant oomycetes (e.g. *Plasmopara halstedii, Pseudoperonospora cubensis* or *Bremia lactucae*), such a system has not been established yet for *P. viticola*.

A specific problem in the case of grapevine and *P. viticola* is the perennial nature of the host plant which makes infection studies under controlled conditions more complicated and time consuming than with seedlings of annual crop plants (e.g. *P. halstedii* / sunflower). A bioassay based on leaf discs inoculations could ease these constrains, although it is known from sunflower that resistance reactions towards downy mildew can be organ specific (Radwan *et al*., 2005) and experiments with detached leaves may

not reflect the infection behavior of whole plants (Spring *et al*., 1997). However, in *Vitis* the infection of *P. viticola* usually starts through the stomata of leaves. This makes it very likely that *in vitro* infection experiments are a realistic basis for the situation in nature, and this kind of approach has been found more practical than greenhouse or field studies (Kast 2001, Deglène-Benbrahim *et al*., 2010). We therefore aimed to use a standardized leaf disc bioassay for the differentiation of *P. viticola* strains on *Vitis* genotypes with different resistance levels. This technique allowed us to perform a fast and practical screening for interesting strains bearing high virulence against resistant genotypes or some degree of fungicide resistance. Similar approaches to evaluate host responses and fungicide resistance were reported by some authors (Peressotti *et al*., 2010, Genet & Jaworska, 2013, Rouxel *et al*., 2013). In contrast to the leaf disc bioassay recently reported by Rouxel *et al*., (2013), we decided to use a higher inoculum density in order to avoid inconsistent infection rates due to variable performance of sporangia from one experiment to the next. Moreover, we preferred inoculation on water instead of filter paper, because the floating culture provided the most homogeneous contact of the plant surface with the medium in which the fungicides were applied. We decided not to evaluate the host reaction on counts of the number of sporangia per leaf disc because this quantitative approach highly depends on homogeneity of the leaf structure (e.g. size of intercostal area, pubescence etc.) which affects the area of inoculation with the applied drop. Moreover, measuring the quantity of sporangia does not take into account the reaction of the host.

1.4.2 Host selection and resistance response

The selection of the host genotypes was guided by the idea of having a generally susceptible host like *V. vinifera* cv. Müller-Thurgau as a control for the efficacy of the infection bioassay. On the other hand, we selected two additional commercially available grapevine cultivars and three wild *Vitis* species. The cultivars Regent and Cabernet Cortis were known from breeding studies to show moderate to strong resistance against downy mildew. The resistance in Regent was attributed to the QTL *Rpv3* (VIVC 2015) while in Cabernet Cortis the resistance is assumed to be associated with *Rpv10*, deriving from its Asiatic ancestor *V. amurensis* (Schwander *et al*., 2012). Of the used wild *Vitis* species, the QTLs *Rpv5*, *6, 9* and *13* were reported from *V. riparia* (Marguerit *et al*., 2009, Moreira *et al*., 2011) while no information was available for *V. vinifera* ssp. *sylvestris* and *V. rupestris*. In contrast to previous infection studies with *P. viticola* on grapevine (e.g.

Bellin *et al*., 2009, Díez-Navajas *et al*., 2008, Rouxel *et al*., 2013), we tried to avoid inconsistency in the host reaction eventually stemming from genetic heterogeneity of the pathogen inoculum. Therefore, we cloned and maintained single sporangium strains of each of the field isolates tested. This allowed, for the first time, to assess the pathogenic diversity within samples collected from a single plant.

Genetic homogeneity might be better achieved by using single zoospore strains. However, its generation is quite challenging due to the very low success rate (less than 0,1 %) compared to 8 % with single sporangium inoculations. Furthermore, microsatellite analysis of thirteen of our single sporangium isolates did not give hints for the presence of mixed genotypes in a sample (see chapter 2).

In general, the selected host genotypes showed the expected resistance levels. So, the cultivars Regent and Cabernet Cortis, both known to be tolerant against downy mildew (Welter *et al*., 2007, Spring 2005), showed moderate to strong resistance reactions. Although Regent contains a resistance related loci, as mentioned before, it mostly allowed stronger sporulation than Cabernet Cortis. On the other hand, the resistance level strongly depended on the pathogen sample assessed. For example, Cabernet Cortis was less resistant than Regent to single sporangium strains from isolates 1117 and 1136 while Regent was fully susceptible to single sporangium strains from isolate 1137 which could barely sporulate on Cabernet Cortis. This indicates that resistance in these cultivars might be based on different mechanisms.

In the group of wild *Vitis* genotypes, the accession of *V. vinifera* ssp. *sylvestris* was the least resistant followed by *V. rupestris* and the most resistant was *V. riparia*. Nevertheless, the degree of resistance found in the used accession of *V. vinifera* ssp. *sylvestris* was higher than expected from field observations in other research groups (Töpfer, Zyprian pers.com.). Microsatellite analysis of our plants showed some deviations in comparison to accessions of *V. vinifera* ssp. *sylvestris* from the Rhine valley (data not shown), so that we cannot rule out a possible resistance introgression through a hybridization event in the ancestry of our material. Anatomical differences in the stomata architecture may explain the lower susceptibility of some wild *Vitis* species (Jürges *et al*., 2009), while physiological adaptations might lead to an early arrest of the infection on the resistant cultivars (Kortekamp & Zyprian 2003, Alonso-Villaverde *et al*., 2011, Malacarne *et al*., 2011). It has been reported that zoospores might

fail to track the stomata on *V. vinifera* ssp. *sylvestris* leaves, hence having a reduced chance for colonization (Jürges *et al*., 2009). The North American species *V. riparia* and *V. rupestris*, on the other hand, possess a mechanism to hinder the invasion of *P. viticola* by stopping the development of the pathogen in the substomatal cavity (Unger *et al*., 2007, Díez-Navajas *et al*., 2008). This is usually accompanied by strong necrosis typical for the activation of defense reaction after penetration of host cells by the pathogen (Díez-Navajas *et al*., 2008, Polesani *et al*., 2010, Alonso-Villaverde *et al*., 2011). Nevertheless, we found at least some degree of sporulation on the highly resistant genotypes with all five pathogen populations tested. Müller-Thurgau was the only genotype showing no hypersensitive reaction against any of the tested strains, thus underlining its suitability as a positive control in infection bioassays with *P. viticola*.

The identification of specific host-pathogen combinations with clearly distinct resistance reactions is a helpful result of this study for future attempts to unravel structural, physiological or molecular mechanisms of compatible or incompatible reactions. The fact that no host genotype was found fully resistant to all tested strains of *P. viticola* implies that future breeding should aim on the appropriate integration of multiple resistance genes in cultivars for increasing the durability of resistance against such a variable pathogen (Eibach *et al*., 2007, Gessler *et al*., 2011). Strains which expressed high virulence in the current study may be used for screening of additional sources of resistance in wild species.

1.4.3 Phenotypic diversity in *P. viticola*

On the pathogen side, the study revealed a high pathogenic diversity between accessions from different geographical origins (France, South Baden, North Württemberg), but also depending on the host cultivar from which the isolate was collected (e.g. isolates 1136 and 1137 are from the same geographic region, but from different host cultivars). The latter shows that host resistance selectively contributes to the formation of *P. viticola* subpopulations differing in pathogenicity. This is in line with findings of Rouxel *et al*., (2013) who reported that pathogen samples collected from the same host genotype in different geographic regions where genetically more similar that samples from different hosts of the same region.

Even more remarkable seems the diversity we found between the single sporangium strains of each field isolate. This corroborates the high genotypic diversity found in molecular genetic studies of *P. viticola* (Gobbin *et al*., 2005, 2006, Chen *et al*., 2007, Rumbou & Gessler 2007, Matasci *et al*., 2010). The report that even a single lesion could be caused by two or more different genotypes (Gobbin *et al*., 2003a) is supported by the identification of mating types which need to share the same intercostal area of the leaf to achieve sexual reproduction in a heterothallic oomycete (Wong *et al*., 2001).

The results show that pathotypes studies in *P. viticola* unavoidably rely on cloned isolates, even if their generation is still very time consuming and laborious because of the low ratio of successful infections with single sporangia. This accounts also for phylogenetic studies which still mostly rely on bulk samples (e.g. Rouxel *et al*., 2013). Particularly the fungicide tests revealed that the results obtained with field isolates did not mirror the diversity found in the cloned strains (e.g. field isolate 1117 versus strain 1117-A7 with dimethomorph or field isolate 1137 versus strain 1137-A10 with metalaxyl-M). The proportion of resistant sporangia in a field population may be so small that infection in the bioassay fails, indicating a false negative result due to the low infection pressure exerted by these sporangia. It is noteworthy that all 5 *P. viticola* field isolates harbored pathotypes showing resistance to either metalaxyl-M, dimethomorph or both fungicides. The dominance of fungicide resistant strains in 1191 may be indicative of a selection in the population due to intensive and multiple applications of the same fungicides. A recent study on *Peronospora tabacina* has shown that changes in the chemical control of tobacco blue mold can lead to a genotypic shift in the pathogen population within few years (Spring *et al*., 2013). Currently solutions used by wine growers are the combination of fungicides with different modes of action or an alternate application, but the selection pressure seems to still be present (Corio-Costet *et al*., 2011) what emphasizes the urgent need for breeding new cultivars.

Chapter 2

Broadening of the system to characterize *Plasmopara viticola* strains

Most of the results presented in this chapter are part of the B.Sc thesis of Markus Kaiser under my co-supervision.

2.1 Introduction

Working with genetic homogeneous material has been many times underestimated in oomycetes research. Our system revealed that single sporangium strains from a field isolate produce different infection reactions on selected hosts (Gómez-Zeledón *et al*., 2013). This underlines the heterogeneous nature of the field isolates and emphasizes the importance of strain characterization. The previously established characterization system (chapter 1) allowed not only the assessment of the variability in downy mildew strains but also gave us information of interesting reactions on specific host-pathogen combinations. Using cultivars of the grapevine *V. vinifera* as well as wild *Vitis* species constituted a very useful system to detect particularities in isolates from different regions and collected on different hosts. Our published system has been successfully used by other authors to evaluate the virulence of *P. viticola* strains confirming its suitability for characterization (Li *et al*., 2015).

The gene reservoir present in the wild species is considered an important source for resistance genes for the breeding programs (Gessler *et al*., 2011). *Vitis*, a genus with more than 60 species, has wild members in regions all around the north hemisphere. Numerous species have been identified in North America and Asia, constituting an important source for downy mildew resistance. In the search for characteristic infection reactions combining hosts with different susceptibility and strains with various levels of virulence, North American and Asiatic species were tested. The presence of resistance loci in Asiatic species has already been confirmed at least in *Vitis amurensis* (Schwander *et al*., 2012). *Vitis jacquemontii* has shown interesting reactions (Jürges *et al*., 2009) and needs to be considered. The North American *Vitis* species have proven to be important for the characterization showing a broad range of resistance to the pathogen (Staudt & Kassemeyer 1995). The possibility to improve the capacity to detect finer differences between strains by incorporating new host species to the characterization system was studied.

The capacity to discriminate between strains with different virulence depends not only on the host species but also on the evaluation system. After testing new isolates from different regions, difficulties were found to distinguish between strains with very similar infection reactions (Kaiser 2015). Attempts were made to improve the capacity of genotypes discrimination considering the reaction of the plant (necrosis) and the reaction of the pathogen (sporulation) using different symbols.

2.2 Material and methods

2.2.1 Asiatic and North American *Vitis*

In order to broaden the phenotypic characterization system, Asiatic and North American *Vitis* were selected to test the infection reaction of strains with different virulence. One strain from each of the five field isolates was selected based on their characteristic reaction on the six previously tested grapevine genotypes. Four Asiatic and four North American species were selected (Table 2.1). The reaction on *Vitis jacquemontii* (Vjaq-01, ID: 5883) was studied to test its suitability for the bioassay. Leaves and cuttings were kindly provided by Dipl.-Biol. Viktoria Tröster from the Botanical Garden, Karlsruhe Institute of Technology (KIT). Mature plants growing outdoors in the botanical garden were selected to harvest the leaves for the test. The reaction against the pathogen was also tested in leaves from *Parthenocissus tricuspidata* and *P. quinquefolia*, two species belonging to the Vitaceae family, growing in the botanical garden of the University.

Table 2.1: Host genotypes used in the new characterization system.

Groups	Host genotypes
Group 1: *Vitis vinifera*	Müller-Thurgau (MT), ID: FR3 vg
	Regent (REG), ID: rpv.3
	Cabernet Cortis (CAB), ID: FR680
	Vitis vinifera L. ssp. *sylvestris* (SYL), WBI
Group 2: North Amerian species	*Vitis rupestris* (RUP), Vrup-01, ID: 5888
	Vitis riparia (RIP), Vrip-01, ID: 6548
	Vitis cinerea (CIN), Vcin-01, ID: 6128
	Vitis aestivalis (AES), Vaes-01, ID: 5911
Group 1: Asiatic species	*Vitis coignetiae* (COI), Vcoi-01, ID: 6542
	Vitis amurensis (AMU), Vamu, ID: 6540
	Vitis davidii (DAV), Vdav-01, ID: 6544
	Vitis betulifolia (BET), Vbet-01, ID: 6126

Leaf selection was based in the criteria already mentioned (1.2.1) and the bioassay was performed as described in 1.2.2 with some adjustments: 2000 sporangia were inoculated and five leaf discs of each genotype were used. Experiments were repeated at least once. Cuttings of the selected species were planted in pots and kept in the greenhouse for further experiments under the already mentioned conditions.

2.2.2 Improvement of the phenotypic characterization system

Due to the fact that different host reactions were not possible to be distinguished using the established methodology to assess the phenotypic variability of the *P. viticola* strains, additional experiments were carried out (Bachelor thesis, Markus Kaiser). It was observed that different reactions were placed in the same category since the protocol gave the same weight to sporulation and necrosis. The following attempt separated the necrosis from the sporulation using the same code system for describing the level of sporulation of selected isolates, but characterizing the necrosis using the following symbols: (+++) strong, (++) moderate, (+) weak and ( ) absence of necrosis (Fig. 2.1). This methodology was applied to characterize the reaction of five selected strains on the *V. vinifera* cultivars and on the North American and Asiatic *Vitis* species. Leaf disc assays were performed as in section 1.2.2.

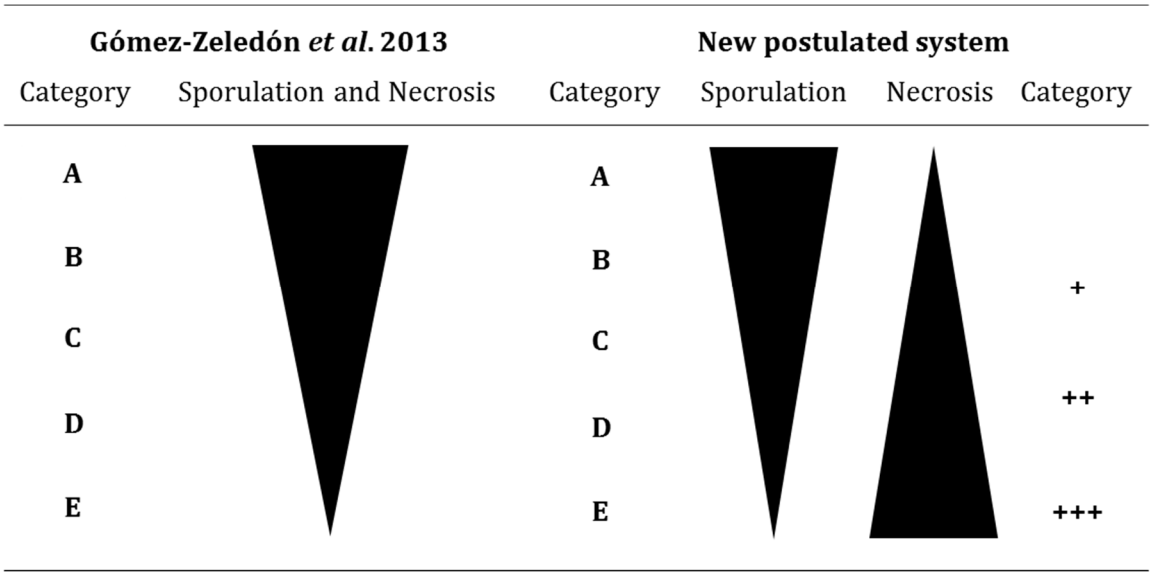

Figure 2.1: Diagram showing the already published evaluation system with sporulation and necrosis together (Gómez-Zeledón *et al*., 2013) and the new postulated system considering sporulation and necrosis in two separated categories.

2.3 Results

2.3.1 Asiatic and North American *Vitis*

Just one out of the four selected Asiatic *Vitis* genotypes, namely *V. davidii*, was able to completely hinder the sporulation of the five selected strains (Fig. 2.2). In contrast, in *V. betulifolia,* all strains achieved a strong sporulation, similar or even stronger to that produced in the susceptible cultivar Müller-Thurgau. In *V. coignetiae*, the sporulation reached category B in all the strains except for 1137-C20, which rarely sporulated in this genotype (reaction type D). Contrary to the expectations, the infection in *V. amurensis* was significantly stronger as presumed (category C) characterized by a moderate necrotic reaction and with a similar behavior as *V. coignetiae*, ranking the isolate 1137-20 as the weakest one (Fig. 2.2).

Regarding the North American species, *V. riparia* was confirmed as highly resistant followed by *V. aestivalis*. In both of them the highest infection category achieved was D, and almost no sporulation was found in any of the tested strains. On *V. cinerea*, the five strains produced a strong sporulation, similar to that in *V. betulifolia*, while the sporulation on *V. rupestris* was moderate.

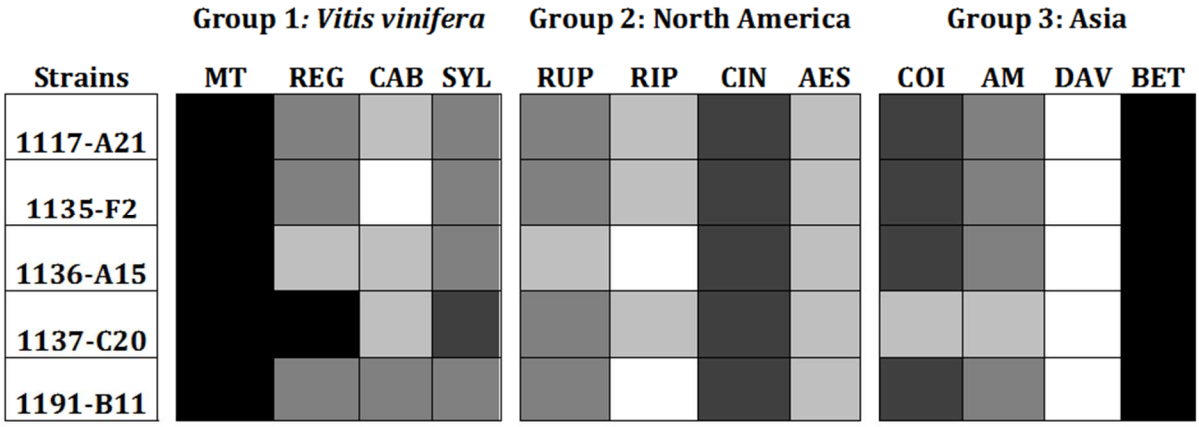

Figure 2.2: Reaction test of the five selected strains. The host genotypes are divided in three categories, *Vitis vinifera* group, North American wild species and Asiatic wild species. Infection reaction: A ■ Profuse sporulation; B ■ Moderate sporulation; C ■ Strong necrotic reaction with scattered sporulation; D ■ Rare sporulation with light necrotic reaction; E □ No sporulation or necrotic reaction.

From the tested species, the one with the higher density of trichomes was *V. jacquemontii*. It was observed that the drops of sporangia suspension applied on leaves of this species were not able to spread in the surface and most of them remained over the trichomes after two days. Subsequent experiments (Bachelor thesis, Markus Kaiser) confirmed this observation. The results obtained with *V. jacquemontii* were very inconstant. In all the strains it was observed that in a few discs, in cases where the trichomes were not so dense, slight sporulation was present. These results were hard to reproduce in subsequent experiments and we decided not to include this species in the genotyping system.

The two species from the genus *Parthenocissus* suffered, surprisingly, a considerable infection when inoculated with the downy mildew strains, allowing the pathogen to sporulate. Due to the lack of resistance in these species, and difficulties in their handling, no further analyses were performed on them.

2.3.1 Improvement of the phenotype characterization system

The assessment of sporulation and necrosis together showed to have its limitations. After phenotyping many different strains from various field isolates with the published system, it was detected that different host reactions were placed in the same category leading to a loss of valuable information (Data not shown). This problem was overcome by independently evaluating sporulation and necrosis (Tab. 2.2/Fig 2.3).

This system would allow a better assessment of the small differences between similar infection reactions. It would be especially useful in cases where it is important to distinguish if an isolate achieved sporulation on a host or just produced a little necrosis (Fig. 2.4). It also improves the categorization of reactions where the necrosis is very strong but the sporulation is low, which in the published system would be scored with a stronger value (Fig. 2.4).

Table 2.2: Description of the new proposed system for the phenotypic characterization of *P. viticola* strains according to the infection reaction produced on leaf discs 10 days after inoculation. Sporulation is designated using colors/letters and necrosis using the symbol (+).

Code	Category	Reaction description
	A	Very strong sporulation (not limited to the inoculation site)
	B	Strong sporulation (limited to the inoculation site)
	C	Moderate sporulation (Scattered sporulation)
	D	Weak sporulation (Single sporangiophores)
	E	No sporulation

+++	Strong necrosis	Defined necrotic area fully covering the infection site
++	Moderate necrosis	Defined necrotic area partially covering the infection site
+	Weak necrosis	Individual necrotic points apart from each other
	No necrosis	

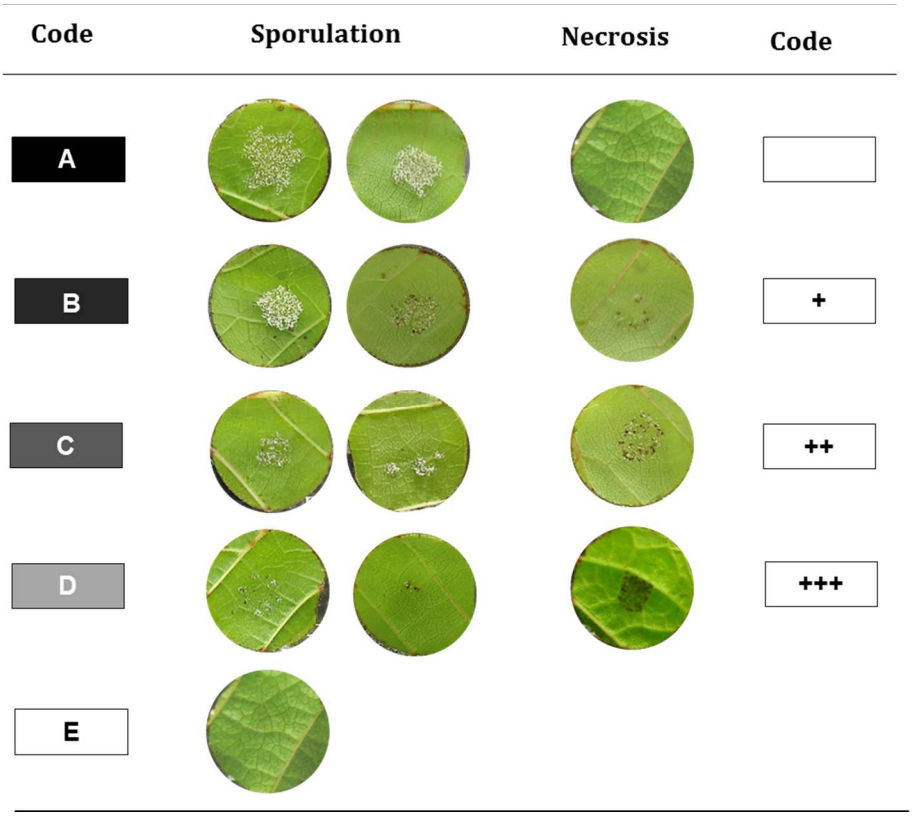

Figure 2.3: Photographic characterization of the reaction categories according to the sporulation (A-E) and the necrosis (+, ++ and +++) of the new proposed system. Pictures were taken by Markus Kaiser.

Strain/ host	Reaction	Category (Gómez-Zeledón *et al.* 2013)	Category (New postulated system)
1191-B11 *V. coignetiae*		D	D +
1191-B11 *V. amurensis*		D	E +
Isolate 1276 *V. vinifera* ssp. *sylvestris*		D	C +
1137-B11 *V. amurensis*		C	E +++
Isolate 1274 *V. rupestris*		C	D +++
1191-B11 *V. coignetiae*		C	E ++

Figure 2.4: Comparison of assessment systems in difficult cases. Infected leaf discs 10 days after inoculation are compared. Pictures were taken by Markus Kaiser.

The reaction produced by the five strains on the selected host plants of Fig. 2.2 was evaluated using the new proposed system. Results are shown in Fig. 2.5. The system showed a new perspective, making it clear that if a color is present it implies a certain degree of sporulation, while the level of necrosis is easily scored with another symbol. The case of *V. amurensis* is an example of this. While in the published system the strains are scored with a reaction type C or D, in the new system all of them are rated as E making clear that no sporulation was present on this species and differentiating them just in the degree of necrosis. In *V. riparia*, as well, the new system explained better the difference between infection reactions. Similar to *V. amurensis*, this species did not allow any sporulation of the five strains but presented a certain degree of necrosis.

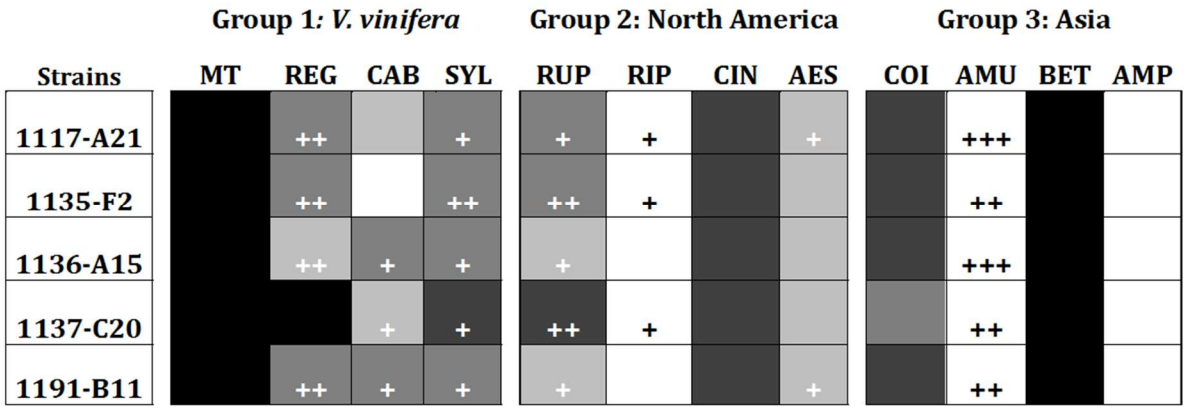

Figure 2.5: Reaction test of the five selected strains according to the new proposed system considering sporulation and necrosis apart. The host genotypes are divided in three categories, cultivars of *V. vinifera*, North American wild species and Asiatic wild species. *Sporulation*: **A** ■ Very strong sporulation not limited to the inoculation site; **B** ■ Strong sporulation limited to the inoculation site; **C** ■ Moderate and scattered sporulation; **D** ■ Weak sporulation with single sporangiophores; **E** □ No sporulation. *Necrosis*: Strong necrosis (+++); Moderate necrosis (++); Weak necrosis (+); No necrosis ( ).

2.4 Discussion

2.4.1 Asiatic and North American *Vitis*

Asiatic *Vitis* species are known as important sources of resistance against *P. viticola* (Wan *et al.*, 2007, Schwander *et al.*, 2012). Thirty five, from the more than 60 known species from the genus *Vitis*, have their origin in China and many of these wild grapes have been used for wine production recently due to their desirable characteristics (Wan *et al.*, 2008) which confirms their potential for breeding combined with European species. *Vitis betulifolia* was an interesting case, which, contrary to our assumptions, responded against all the tested strains like our susceptible cultivar Müller-Thurgau. In some cases, the spread of the infection on the inoculated discs was surprisingly faster and stronger than in Müller-Thurgau. Due to its high susceptibility, leaves of this species would be suitable to be used as a positive control on the leaf discs experiments or even to perform the subculture of the isolates or for cloning. No information was found in the literature about the downy mildew resistance of this species. Another Asiatic species previously tested, but not included in this study, namely *Vitis thunbergii*, exhibited a strong infection when inoculated with different *P. viticola* strains, as well. This demonstrates that a wide range of degrees of susceptibility against this pathogen can be found in the Asiatic species. *Vitis coignetiae* and *V. amurensis* showed an intermediate

resistance reaction, similar to that of *V. riparia* and *V. vinifera* spp. *sylvestris*. Interestingly, no difference was found between the response of these species against the five selected strains except of 1137-C20 which was the less virulent. This strain was previously known for its strong sporulation in the tolerant cultivar Regent, showing that different mechanisms are responsible for the resistance in the Asiatic species. In contrast to the reported information available on *V. coignetiae* (Jürges *et al*., 2009), the five strains tested achieved moderate sporulation on this species, what could be attributed to intraspecific differences of susceptibility, a phenomenon that has already been observed (Yu *et al*., 2012).

The fact that all the strains showed the same reaction might be evidence that these European isolates haven't been in contact with Asiatic *Vitis* species in their evolutionary history, thus preventing them to develop mechanisms to overcome the effect of the preexistent resistance of these plants. The lack of difference in the reaction on Asiatic species could also indicate that the five isolates have a common European ancestor and that the divergence took place not so long time ago.

Even though the presence of a resistance locus have been reported on *V. amurensis*, (*Rpv10*) it has been shown that there is a strong variation of downy mildew resistance between accessions of this species (Blasi *et al*., 2011, Yu *et al*., 2012, Schwander *et al*., 2012, Venuti *et al*., 2013). This underlines the importance of a careful selection of accessions of wild species for phenotyping. A strong necrotic reaction with no sporulation on *V. amurensis* implies a high level of resistance of the tested genotype in the present study. This Asiatic species has been previously characterized for suppressing sporulation while reacting with strong necrosis (Boso & Kassemeyer 2008). The only Asiatic species in which none of the selected strains of *P. viticola* was able to produce either sporulation or necrosis was *V. davidii*. Further experiments using fluorescence microscopy showed that hyphal growth took place on the leaves of this species even though infection symptoms were absent, demonstrating that zoospores can reach the stomata, germinate and grow inside the leaves. Contrary to these results, other authors have reported the development of *P. viticola* infections with sporulation on *V. davidii* (Yu *et al*., 2014, Liu *et al*., 2015). Further studies using molecular markers and morphology by Dr. Wei (Missouri Botanical Garden, and Flora of China), showed that this species originally designated as *V. davidii*, corresponds to another Asiatic species from the same family, namely *Ampelopsis japonica*, which would explain the

discrepancies found. Even though, the results found with this species are of special interest, since other closely related species to *V. vinifera* are potential sources of resistance genes against downy mildew (e.g. *Muscadinia rotundifolia,* Merdinoglu *et al*., 2003).

The North American species are known to present high resistance against *P*. *viticola* due to their evolutionary history. Nevertheless, just one species namely *V*. *riparia* was able to completely block the sporulation of the selected strains, contrary to the observations made in previous years. Several loci related with resistance have been reported to be originated from this species (Marguerit *et al*., 2009, Moreira *et al*., 2011). On *V*. *rupestris*, moderate sporulation and necrosis was observed in all cases tested here. This partial resistance might be conferred by an arrest of the pathogen growth on the early stages of the infection and anatomical barriers in the stomatal structure (Denzer *et al*., 1995a, Kortekamp & Zyprian 2003, Jürges *et al*., 2009). Sporulation in *V*. *riparia* has already been reported by other authors (Díez-Navajas *et al*., 2008) and in our first experiments, but it has also been reported that the reaction to the pathogen in this species might vary depending on the accession (Staudt & Kassemeyer 1995). Inoculum concentration, sporangia viability and environmental conditions might play an important role between no sporulation at all or a slight sporulation on this species. In contrast to the results of Unger *et al*., (2007), sporulation was found in all the tested strains on *V*. *rupestris*. On *V*. *aestivalis* the infection reaction was very similar, but in this case all strains were able to weakly sporulate. Both *V*. *cinerea* and *V*. *aestivalis* had been reported as moderately resistant against downy mildew (Staudt & Kassemeyer 1995), nevertheless the fact that the five strains moderately sporulate on *V*. *cinerea* indicates once again that not all accessions of a resistant species show the same response.

The inability of *P. viticola* to successfully infect the Asiatic species *Vitis jacquemontii* is attributable to a mechanical barrier imposed by the dense coverage of trichomes found in the lower side of the leaves. This barrier avoided the direct contact of the zoospores with the stomata. Staudt & Kassemeyer (1995) reported that the hairiness of the leaves of some wild *Vitis* species impeded an infection of downy mildew. The fact that sporulation was found in some of the leaf discs suggests that there might not be a strong physiological defense in this genotype, and that its resistance is mainly due to this mechanical protection. This result contrasts with the findings from Jürges *et al*., (2009) where the formation of long surface mycelia is reported. On such hairy leaves the

formation of this kind of mycelium seems not plausible and deserves reinvestigation. In microscopy studies we were never able to observe long external mycelia, but just germinated spores with normal germ tubes that never got into the stomata due to the hairy barrier.

Even though it is commonly believed that *P. viticola* is not able to infect species out of the genus *Vitis*, previous observations showed that the pathogen can colonize and sporulate on two other Vitaceae species, *Parthenocisus tricuspidata* and *P. quinquifolia*. In contrast, the findings of Rouxel *et al*., (2012) suggest that *P. viticola* strains found in resistant cultivars or on different Vitaceae species, due to a strong host specialization, would not be able to infect the traditionally susceptible cultivars. In spite of that, our strains, whether they were isolated from susceptible or resistant *Vitis* genotypes, were able to infect a wide variety of hosts.

2.4.2 Improvement of the phenotypic characterization system

Sporulation and necrosis are essential features for the characterization of an infection reaction on a biotrophic pathogen. The level of sporulation achieved by a strain is related to its virulence but it is not the only important characteristic. Regular phenotyping systems are based on the amount of sporangia, and do not consider the necrosis or evaluate it together with the sporulation (Bellin *et al*., 2009). The presence of resistance genes in the host genome and the development of necrosis are related (Gindro *et al.*, 2003, Bellin *et al*., 2009, Blasi *et al.,* 2011). The new proposed system gives weight to the necrosis, but also allows the separation of this character from the sporulation, facilitating the interpretation of the results. Necrosis formation suggests a strong host reaction against the pathogen, but also indicates a certain level of susceptibility (Blasi *et al.,* 2011), which is not considered when sporulation is evaluated alone. Occurrence of necrosis due to a hypersensitive reaction (HR) suggests the recognition of the pathogen and the activation of the plant defense reaction (Gindro *et al.,* 2003). Necrosis is recognized as a good indicator of grapevine resistance and has been previously considered when evaluating downy mildew resistance, but very roughly (Deglène-Benbrahim *et al*., 2010) and not so detailed as in this study. The absence of necrosis implies a compatible reaction in case of sporulation or a nonhost reaction in case of the absence of it. The kind of protection to the plant conferred by the nonhost resistance is more durable than the one conferred by R genes. Especially type I nonhost

resistance (preformed plant defense mechanisms) would be ideal for breeding, because it does not involve HR (Mysore & Ryu 2004).

Small discrepancies between the results of two years in a bioassay are unavoidable. The system is affected by many sources of variation like leaf age, physiological development of the plant, particular weather conditions, presence of other not detected pathogens, and others. An example reported by Steimetz *et al*., (2012) showed the influence of the leaf age on the resistance response against *P. viticola*, being older leafs more responsive and less susceptible than younger leaves to the infection. It has also been reported that the seasonal development affects the production of stilbenes during a downy mildew infection in grapevine (Gindro *et al*., 2012). From the side of the pathogen there are as well many variation sources like age of sporangia, viability, physiological state, nutritional state according to the leaves used for subculture, and others. It makes clear that comparison of results from different years should be avoided. However, beside small differences, a general trend is easy to recognize and the characterization of the strains is possible and it represents, due to the lack of information in this area (Kamoun *et al*., 2015) an important step to improve breeding strategies.

Microscopy performed by Markus Kaiser for his bachelor thesis showed similar results to those reported in the literature (Unger *et al*., 2007, Jürges *et al*., 2009), where shortly after penetration in the substomatal cavity, resistant hosts are able to arrest the pathogen development. Although numerous studies have been conducted on the relationship between *P. viticola* and *Vitis* species in the last years, still many questions remain unanswered. Which plant genes are able to hinder the hyphal growth on the resistant species? How do these genes exert their activity? Is it one gene or is it a combination of many genes that confers resistance? Which factors enable *P. viticola* strains to overcome the defense reaction? In order to assess these questions, molecular and phytopathological studies are needed to unravel the mechanisms behind this complex relationship. The idea to use a phenotypic characterization system including species from different geographical regions enabled us to screen *P. viticola* strains in terms of their ability to overcome many different defense mechanisms. Having a system ranging from fully susceptible to fully resistant species confers the possibility to characterize strains with a wide range of pathogenicity.

Chapter 3

Molecular characterization of single sporangium isolates

3.1 Introduction

Populations of *P. viticola* are characterized by a high genetic variability, which makes its control a challenging task (Stark-Urnau *et al*., 2000, Gessler *et al*., 2001, Peressotti *et al*., 2010). Many molecular markers have been developed in the previous years, aiming for a better understanding of the population dynamic of this pathogen. For the study of genetic variation in oomycetes, specific markers that are polymorphic at the intra-specific level are necessary, but their development is often much more laborious than the search for markers that are only species specific (Giresse *et al*., 2010). Microsatellites, or simple sequence repeats (SSRs), are abundant sequences with a high level of polymorphisms that are dispersed throughout the genomes of most eukaryotes. This kind of markers have been widely used for population dynamic studies and DNA fingerprinting due to its co-dominant nature, and for being locus specific (Gupta *et al*., 1996). SNPs (single nucleotide polymorphism), on the other side, markers are characterized by a single base change in a specific DNA region, where usually two alternative nucleotides (biallelic) might be present in a certain position (Vignal *et al*., 2002). To date, 42 microsatellites (SSRs) and 8 SNPs markers have been described for *P. viticola* (Gobbin *et al*., 2003a; Matasci *et al*., 2010; Delmotte *et al*., 2011; Rouxel *et al*., 2012). These molecular tools improve the genotyping efficiency for this important pathogen and allow the design of new genetic studies. The usage of single nucleotide polymorphisms on oomycete population studies has proven to be effective in *P. viticola* (Delmotte *et al*., 2011) as well as in *Plasmopara halstedii* (Giresse *et al*., 2007, Delmotte *et al*., 2008, Viranyi & Spring 2011). Microsatellites markers (SSRs) have demonstrated to be useful tools for genotyping *P. viticola* isolates using allele patterns (Gobbin *et al*., 2003ab, Delmotte *et al*., 2006, Matasci *et al*., 2010, Roatti *et al*., 2013).

In this study we aimed to achieve a molecular characterization of selected strains of *P. viticola*, which would permit a better handling and understanding of their development in population studies. The five selected strains from chapter 2 were analyzed at the molecular level in this chapter. Eight strains from the field isolate 1191

were examined as well. We focused on the molecular level, looking for a confirmation that would explain the high variability observed in chapter 1 and 2 at the phenotypic level. After the establishment of a system to characterize strains based on their interaction with hosts of different susceptibility, our next step was to characterize those outstanding strains at the molecular level for further studies. The quite different response observed on sister strains in chapter 1 will be contrasted with new results at the molecular level to confirm the assumptions that different genotypes are present in a field isolate. Finally, the homogeneity of single sporangium strains assumed by their stability through infection tests needed to be confirmed using molecular tools.

3.2 Material and methods

3.2.1 Material selection and DNA extraction

The field isolate from which the highest number of single sporangium strains was obtained in previous experiments was 1191. Eight strains of this field isolate were selected to examine the genotypic diversity. The previously selected strains (chapter 2), one from each of the other four available field isolates (1117, 1135, 1136 and 1137), were incorporated in the molecular analysis performed in this chapter. Sporangia were collected and frozen using the vacuum system mentioned in chapter 1. DNA was extracted according to a standard protocol from the laboratory (Frey & Spring 2015). After measuring the amount of DNA and dilution to a concentration of 30 ng/µl, PCR was performed in a pecStar 96 Universal Thermocycler (PeqLab Biotechnology, GmbH, Erlangen, Germany) to amplify the desired regions. The quality of the PCR products was checked using microchip capillary electrophoresis (MultiNa, Shimadzu, Duisburg, Germany). The Red*Taq* Mastermix was standardly used for PCR amplification (Table 3.1).

Table 3.1: Standard PCR amplification reaction used with the Red*Taq* Mastermix.

Component	**Manufacturer**	**Volume**
Red*Taq* Mastermix	Genaxxon Bioscience, Ulm	6 µl
Specific primer [10µM]	Sigma-Aldrich GmbH, Munich	each 1 µl
DEPC-treated H_2O	Gibco, Paisley UK	3 µl
DNA		1 µl
Total volume		12 µl

3.2.2 Single Nucleotide Polymorphisms (SNPs)

Eight known species-specific markers obtained from an expressed sequence tag (EST) library (Delmotte *et al.*, 2011) were used to study the variability within a field isolate and between strains from different field isolates. Isolate 1191 was selected, from which 8 single sporangium strains were available. The five selected strains used in chapter 2 were analyzed in the same way for comparison. PCR with primers listed in Table 3.2 was conducted using the conditions reported in Table 3.3. When the quality of the PCR products was satisfactory, samples were prepared for sequencing. The EZ-Sequencing

service from the company Macrogen (Amsterdam, the Netherlands) was chosen for this purpose. Each sample was prepared in a 10 µl final volume including 6,5 µl of DEPC treated water, 2,5 µl of the desired primer (forward or reverse) and 1 µl of the PCR product in a 1,5 ml micro centrifuge tube. Sequences obtained from each isolate were aligned using the software BioEdit (V7.0.9, Ibis Biosciences, Carlsbad, USA) to screen the specific loci where the SNPs should be present. In cases where the baseline noise in the sequencing was very high or the peaks were not clear enough, samples were re-sequenced or the PCR was repeated to assure reliable results. A total of 34 SNPs loci were used to characterize the strains (Table 3.6). Different allele patterns are represented with different colors.

Table 3.2: Sequence of the primers used for the SNPs analysis and approximate size of the expected PCR product (Delmotte *et al.*, 2011). The synthesis of the primers was performed by Sigma-Aldrich GmbH, Munich, Germany.

Primer name	**Sequence 5' → 3'**	**Approximate size (bp)**
Pvi1_F	CCGTGACTCCCTTGTATTCC	494
Pvi1_R	AACGAATAGGGTGCGTAGGA	
Pvi2_F	TAAAGGAGGGCAAGATCAGC	450
Pvi2_R	CGATACCAGCCATACCCAAC	
Pvi3_F	CTCAGGGCGCAGATCAAT	299
Pvi3_R	CAAATCCGTAGGGTTCATGC	
Pvi4_F	CTACATCTCGTCCGAGAAAGG	366
Pvi4_R	ATAGGAATGAGCGGCTGGT	
Pvi5_F	GAGCATTTGCGCGTTGTG	278
Pvi5_R	CGCAGCTCCTTTCCATATTT	
Pvi6_F	GGAAGTATTGGACGACAAGGTC	200
Pvi6_R	TAATAGGGTGAAGCGGGTTG	
Pvi12_F	CTGACGGGCAAGACCATTAC	372
Pvi12_R	GAACACACCAGCACCACACT	
Pvi13_F	CCAAGTCGCAAGCAAGTAAA	638
Pvi13_R	GCGAAAAAGGAAAAATAAGCA	

Table 3.3: PCR conditions used for the SNPs analysis (Delmotte *et al*., 2011).

Step	Temperature	Time
Initial denaturation	94°C	5 min
Denaturation	94°C	50 sec
Annealing	50°C	50 sec
Elongation	72°C	60 sec
Final elongation	72°C	10 min

3.2.3 Short Sequence repeats (SSRs)

The microsatellite experiments were performed at the Julius-Kühn-Institut, Geilweilerhof, in the working group of Prof. Dr. Eva Zyprian and with the collaboration of Dipl.-Biol. Jens Dudenhöffer. From the 42 microsatellite markers known to date for *P. viticola*, eight were selected based on a high polymorphism level. Two multiplex panels of fluorescent-labeled microsatellite primers were used. Simultaneous PCR amplifications were carried out in a final volume of 10 µl containing 10 ng of genomic DNA, 0,25 mM of each dNTPs, 2 mM $MgCl_2$, 10 mM dye-labeled forward and an unlabeled reverse primer (Table 3.4) and 1,5 U Taq DNA Polymerase (AmpliTaq, Gold™, Applied Biosystems, Foster City, CA). A GeneAmp PCR System 9700 was used to perform the PCR reactions under the conditions listed in Table 3.5.

The eight loci were analyzed as follow: PCR products (0,5 µl) generated by two or three different fluorescence dye-labeled primers were mixed with 9,3 µl of formamide and 0,2 µl of GeneScan 600 LIZ Size Standard (Applied Biosystems). The DNA fragments were denatured and size fractionated using capillary electrophoresis on an ABI 3100 Genetic Analyzer (Applied Biosystems, Darmstadt). Subsequently, GeneMapper 4.0 (Applied Biosystems, Darmstadt) was used to score the allele sizes.

Table 3.4: Sequence and fluorescent labels of the primers used for the microsatellite analysis and approximate allele size range. The synthesis of the primers was performed by Sigma-Aldrich GmbH, Munich, Germany.

Primer name	Sequence 5' → 3'	Fluorescent label	Approx. allele size range(bp)
ISA New_F[a]	GGCATGGACGTTGACTCA C	HEX	118-144
ISA_R[a]	GAGAAGTTCCGCCAAGTACA		
CES_F[a]	CTTGTCGGTAGGTAAGCGTG	6-FAM	143-186
CES New_R[a]	CATCAGAATGTTTGTGTGTG		
BER NewF[a]	CAAGCAATGCAATGGTCTTC	ROX	179-185
BER New_R[a]	GGCATCACTCTCTACCTGCTC		
GOB_F[a]	CTTGGAAGTTATACCATGCTACC	6-FAM	210-434
GOB New_R[a]	ATCGCACAGCTTAATGCATATC		
Pv91_F[b]	ACCAGCCTTTGCGAAGATAA	ROX	142-146
Pv91_R[b]	TGAAAGTTACGTGTCGCACC		
Pv137_F[b]	AAGTGGGACACATCAAGCGT	TAMRA	243-256
Pv137_R[b]	TGGCAATAAGTTTATGCCTCG		
Pv143_F[b]	CCTGAATAAAGCAACACGCA	FAM	121-135
Pv143_R[b]	TTGGCAGCAAATTGTACGAC		
Pv144_F[b]	ACCAAGAATCGCACCTAACG	TAMRA	161-192
Pv144_R[b]	GTCTGCCTGTTTGTCGGTTA		

a Gobbin *et al*., 2003a & Matasci *et al*., 2010
b Rouxel *et al*., 2012

Table 3.5: PCR conditions used for the SSRs analysis.

Step	Temperature	Time
Initial denaturation	96°C	15 min
Denaturation	93°C	30 sec
Annealing	60°C	90 sec
Elongation	72°C	60 sec
Final elongation	72°C	30 min

3.3 Results

3.3.1 Single Nucleotide Polymorphisms (SNPs)

Out of the 34 different SNPs loci analyzed, nine possible combinations were found in the 13 strains. Within the field isolate 1191, five different genotypes were found among the 8 tested single sporangium strains, indicating a high genetic variability. Four of the markers (Pvi1, Pvi5, Pvi12 and Pvi13) didn't show differences between the strains of isolate 1191, but they showed differences when compared with strains from other field isolates (Table 3.6). Two strains from different field isolates (1117-A21 and 1136-A15) presented the same allele pattern using the present system. None of the analyzed strains showed signs of a mixture of genotypes (e.g. more than two alleles for a specific loci).

3.3.2 Short sequence repeats (SSRs)

Microsatellites (short elements of repeated sequence motives scattered over the genome) demonstrated to be a very useful tool to study the diversity between genotypes of the same species. Due to the high polymorphism showed, a characteristic pattern was found for the selected strains (Table 3.7). In the 13 analyzed samples, (one field isolate and 8 derived strains plus 4 strains from other fields) the microsatellites presented at least two alleles (BER, ISA, PV91 and PV137) and two of these markers where highly polymorphic with more than five alleles (GOB and PV144) (Table 3.7). Four different genotypes where found when the single sporangium strains from the field isolate 1191 where compared. None of the strains from different field isolates presented the same pattern. In this case, as well as using SNPs, no signs of a genetic inhomogeneity were observed when single sporangium strains were analyzed.

Table 3.6: Identity of single nucleotide polymorphisms (SNPs) localized within eight *P. viticola* EST-derived markers (Delmotte *et al.*, 2011) in the genome of the field isolate 1191 together with its correspondent single sporangium strains (A5, A7, B6, B9, B11, B12, B15 and B18) and four other selected strains (1117-A21, 1135-F2, 1136-A15,1137-C20). Different colors indicate a different allele pattern. Letters indicate the specific base present at that locus. In case of heterozygosis, both bases are presented.

Markers	Strain/Localiz.	1191	1191-A5	1191-A7	1191-B6	1191-B9	1191-B11	1191-B12	1191-B15	1191-B18	1117-A21	1135-F2	1136-A15	1137-C20
	130	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	A	A	A	A
	157	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C	C	C	C
Pvi1	193	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C	C	C	C
	232	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G	G	G	G
	265	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C	C	C	C
	271	C/G	C/G	C/G	C/G	C/G	C/G	C/G	C/G	C/G	A	A	A	A
	292	T/A	T/A	T/A	T/A	T/A	T/A	T/A	T/A	T/A	T	T	T	T
Pvi2	146	T	T	T	T	T	C	T	T	T	C/T	C	C/T	C
	33	C/T	T	C/T	C/T	C/T	C/T	C/T	C/T	T	C	C	C	C/T
	132	C/G	C/G	C/G	C/G	C/G	C/G	C/G	C/G	C/G	G	C	G	G/C
Pvi3	151	G	G	G	G	G	G	G	G	G	G	G	G	G
	174	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	G	A/G	G	A/G
	270	A	A/G	A	A/G	A/G	A/G	A	A	A/G	G	A/G	G	A
	229	C	C	C	C	C	C/T	C	C	C	C	C	C	C/T
	232	C/T	C/T	C/T	C/T	C/T	T	C/T	C/T	T	C/T	C/T	C/T	T
	268	G/C	G/C	G/C	G/C	G/C	C	G/C	G/C	C	G/C	G/C	G/C	C
Pvi4	313	C	C	C	C	C	C/T	C	C	C	C	C	C	C/T
	314	C	C	C/T	C	C	C/T	C	C	C	C	C	C	C/T
	316	T/C	T/C	T/C	T/C	T/C	C	T/C	T/C	T/C	T/C	T/C	T/C	C
	334	C/T	C/T	C/T	C/T	C/T	T	C/T	C/T	T	C/T	C/T	C/T	T
	103	C	C	C	C	C	C	C	C	C	C	C	C	C
	105	G	G	G	G	G	G	G	G	G	G/C	G	G/C	G/C
Pvi5	156	T/A	T	T	T	T	T	T	T	T	T	T	T	T
	166	T/G	T	T	T	T	T	T	T	T	T	T	T	T
	168	A/T	A	A	A	A	A	A	A	A	A	A	A	A
	249	G	G	G	G	G	G	G	G	G	G/A	G	G/A	G/A
	252	C	C	C	C	C	C	C	C	C	T/C	C	T/C	T/C
Pvi6	61	G	G	G	G	G	G	G	G/T	G	G/T	T	G/T	G/T
	73	G	G	G	G	G	G	G	G/A	G	G/A	A	G/A	G/A
Pvi12	149	C	C	C	C	C	C	C	C	C	C	C	C	C
	68	A	A	A	A	A	A	A	A	A	A	A	A	A
Pvi13	196	G	G	G	G	G	G	G	G	G	G	G	G	G
	199	C	C	C	C	C	C	C	C	C	C	C	C	C
	235	T	T	T	T	T	T	T	T	T	T	T	T	T

Table 3.7: Allele size (bp) of the eight SSRs loci (Gobbin *et al*., 2003a, Delmotte *et al*., 2006, Matasci *et al*., 2010, Rouxel *et al*., 2012) from the 13 analyzed samples of *P. viticola* and total number of alleles found. In case of heterozygosis, the size of both alleles is presented.

Strain/Marker	BER	CES	GOB	ISA	PV91	PV137	PV143	PV144
1191	135	149/151	277/379	125/129	145	256	129/137	181/189
1191-A5	135	149/151	277/379	125/129	145	256	129/137	189/191
1191-A7	135	151	277/379	125/129	145	256	129/137	189/191
1191-B6	135	151	277/379	125/129	145	256	129/137	189/191
1191-B9	135	151	277/379	125/129	145	256	129/137	189/191
1191-B11	135	153	367/393	125/129	145/148	256/259	129/137	181/189
1191-B12	135	151	277/383	125/129	145	256	129/137	189/191
1191-B15	135	151	277/379	125/129	145	256	129/137	189/191
1191-B18	135	151	277/379	125/129	145	256	129/137	189/191
1117-A21	135/136	140/153	195/368	125/129	148	256/259	129/132	193/197
1135-F2	135	153	195/381	129	145	256/259	129/132	187/191
1136-A15	135/136	140	195/368	125/129	148	256/259	129/132	193/197
1137-C20	135/136	149	277/373	129	145/148	259	132	185/187
No. of alleles	2	4	9	2	2	2	3	6

3.4 Discussion

In order to study the genetic and genotypic diversity of plant pathogens, different molecular markers have been used by various researchers. AFLPs, ISSRs and RAPDs have been often preferred because they permit a fast screening of various loci within a genome (Stark-Urnau *et al*., 2000, Fry 2001). However, these markers might not be the most appropriate because they are dominant, and not specific to the target. SNPs on the other side are promising markers due to their specificity, their codominant nature and their high resolving power (Delmotte *et al*., 2008). A high polymorphism at the intraspecific level is desirable for population studies and is another advantage of the SNPs (Giresse *et al*., 2007), but the low detection efficiency restricts their application as molecular markers (Liu *et al*., 2012). In the present study the application of these markers in *P. viticola* confirmed their suitability for genotyping in grapevine downy mildew. The allele patterns allowed the detection of different genotypes within a field isolate. However the 34 SNPs loci analyzed were insufficient to differentiate between the two strains 1117-A21 and 1136-A15 although they showed a different infection reaction on Regent. This shows that SNPs markers are suitable for genotyping, but not predictors for pathotypes. The limitation in differentiating between a strain derived from Colmar, France (1117-A21) and one from Pfaffenweiler, Germany (1136-A15) could either imply that some genotypes are distributed over a large geographic area or it indicates that a higher number of markers should be considered to achieve proper separation of genotypes. The dependence on sequencing methodologies and the inefficacy of accurately allele assignment due to sequence noise still limits the application of this technique for the genotyping of large set of strains.

Microsatellites are other species-specific markers suitable for population studies and genotyping on the infraspecific level. This kind of markers represents a very important tool in genetic population studies, as well as in epidemics (Gessler *et al*., 2001, Matasci *et al*., 2010), fungicide resistance (Matasci *et al*., 2008) and genotyping research (Roatti *et al*., 2013). The limiting issue for the use of SSR markers is the allele size determination, due to the small differences in sizes. For this purpose, polyacrylamide gels have been preferred considering its lower cost (Gobbin *et al*., 2001, 2003), however the analysis of the generated PCR products is easier and more accurate through capillary sequencing (in a Genetic Analyzer) with fluorescent labeled primers (Gobbin *et al*., 2003 ab, Matasci

et al., 2010, Rouxel *et al*., 2012). This methodology is more complicated and expensive requiring special equipment and trained personal but provides a higher resolution allowing the analysis of higher sets of data without using toxic substances. Using eight microsatellite markers, we were able to differentiate between downy mildew strains from different field isolates better than using the SNPs. Within the field isolate 1191 four different genotypes were found. When combining the data of SNPs and SSRs it was possible to define each of the tested strains by its characteristic and unique pattern.

The combination of available SNPs and SSRs assures a higher accuracy in genotyping and provides a reliable set of markers for population studies as well as for the characterization of interesting isolates (Delmotte *et al*., 2011). In this study it was demonstrated that such combination provides a very high resolution at the population level, even within strains from a field isolate. This would allow detecting mixed isolates or contaminations. In spite of its advantages, these genotyping protocols are still not able to provide a quick and easy method to differentiate between strains, and are very time and cost intensive.

The high diversity found within a field isolate is in congruence with previous studies (Stark-Urnau *et al*., 2000, Kast 2001, Gobbin *et al*., 2001). It has also been demonstrated that different genotypes might be present even in a single lesion (oil spot) produced by different spores or by heterokaryotic processes in the leaf (Gobbin *et al*., 2003). In the previous chapter it was demonstrated that single sporangium strains from a field isolate are phenotypically different in terms of their ability to infect hosts with different susceptibility. Even their fungicide resistance was different, suggesting a high genetic variability within field isolates. The results in this chapter corroborate that a high genetic variability is behind the phenotypic diversity observed before. This additional evidence confirms the requirement of single sporangium strains to perform studies in such a variable pathogen. If a mixture of genotypes is present in an isolate, the results obtained will be very variable and not repeatable. Even recent studies have relied on bulked samples (Schwander *et al*., 2012) and the results appear vulnerable in the context of the high population diversity already shown (Scherer & Gisi 2006). Single sporangiophore isolates have been alternatively used (Gisi *et al*., 2007, Yin *et al*., 2015) but the possibility cannot be ruled out that parasexual processes might occur in this genus, similar as shown for *Plasmopara halstedii* in sunflower (Spring *et al*., 2006). This

would speak against the assumption that all sporangia in a sporangiophore possess the exact same genetic material.

The capacity of certain strain to overcome the effect of a specific fungicide or to infect a tolerant host might be associated to the presence of a determined allele, (e.g. effector genes), absent in other strains. This would provide a very useful tool for an easier detection of special characteristics and to follow them in the time. It improves as well the ability for early detection of especially dangerous strains in the field and supports the breeding programs facilitating the screening for interesting genotypes. The application of the here presented markers will be the goal of future studies.

Chapter 4

Expression of putative effector genes at early developmental stages in *P. viticola* strains with different virulence

Some of the experiments of this chapter were performed as part of the bachelor theses of Melanie Fröhler, Nele Bendel and Markus Kaiser and the master thesis of Sandra Becker under my co-supervision. Results from this chapter were partially presented on International Workshops held in Vitoria/Gasteiz, Spain (Gómez-Zeledón *et al*., 2014) and in Munich, Germany (Gómez-Zeledón *et al*., 2015).

4.1 Introduction

Biotrophic pathogens establish a very close relationship with their host in their infection course. To be able to live in the apoplast and feed on the plant without activating a defense reaction, biotrophs have developed a wide arsenal of mechanisms. Plants can recognize pathogens based on common substances that reveal the presence of eventual threats. This first level of defense is called PTI (PAMP-triggered immunity) and is effective against a wide range of pathogens (Jones & Dangl 2006). The PAMPs (pathogen-associated molecular patterns) are conserved microbial molecules recognized by the plant innate immune system. Examples of these molecules are flagellin, from bacterial origin, or chitin present in fungal cell walls. Pathogens which overcome this first barrier can successfully infect the plant making use of virulence factors or effectors to suppress the PTI. Effectors are secreted molecules, typically proteins, which interfere with the host immunity. However, plants have developed a second line of defense called effector-triggered immunity (ETI) which directly or indirectly recognizes such pathogens by either detecting the presence of effectors or their activity in the plant. In such a case, effectors are then called avirulence molecules (Avr) because they are recognized by the plants' R-proteins and used as a switch to induce a defense reaction, commonly expressed as a hypersensitive response (Morgan & Kamoun 2007). In the course of time, specialized pathogens have developed effectors capable to disable host defense reprograming the cells and impeding cell death (Panstruga *et al*., 2003). In this case we talk about effector-triggered susceptibility (ETS) since the plant is again defenseless against the pathogen attack (Jones & Dangl 2006, de Jonge *et al*., 2011). This dual function of effectors, either triggering the plant immune reaction or increasing the pathogen virulence have made these molecules the target of

numerous studies trying to improve the understanding of plant-pathogen interactions (Kamoun 2007).

Effectors are not only present in oomycetes, but also in many other organisms. Even though bacterial effectors and their mechanisms have been extensively studied (Hann *et al*., 2010), there are still many open questions about the role and virulence mechanisms of oomycete effectors (de Jonge *et al*., 2011, Bozkurt *et al*., 2012). Efforts making use of bioinformatics tools have enabled the discovery of hundreds of oomycete effectors, but just a few of them have been fully characterized (Kamoun 2006, Fawke *et al*., 2015). Effectors might target different compartments in the host cells and based on this could be roughly divided in two groups: apoplastic and cytoplasmic effectors (Schornack *et al*., 2009). Both kinds of effectors are secreted by means of a specialized infection structure called haustorium, which not only allows the pathogen to extract nutrients from host cells, but also opens the pathogen a door to manipulate the host and successfully infect it (Voegele *et al*., 2001, Panstruga 2003, Panstruga & Dodds 2009, Tyler 2009). The role of effectors is summarized in Fig. 4.1.

Apoplastic effectors fulfill different tasks, mainly in the first contact of the pathogen with the plant. Cell-wall degradation, inactivation of hydrolytic host enzymes and scavenge of potential PAMP molecules belong to them (de Jonge *et al*., 2011). Kamoun in 2006 came out with a detailed catalogue of the known oomycetes effectors and grouped them according to their localization and their biological activity. Apoplastic effectors play a very important role in the establishment of the infection in its first stages, but they might also be key molecules for the plant to recognize the pathogen (Fig. 4.1). Cytoplasmic effectors are characterized by their translocation into the plant cytoplasm. Two families of cytoplasmic effectors have been described for oomycetes, the RXLR and the CRN (Crinkler) family. The RXLR effectors have only been reported on individuals from the Peronosporales, and seem to be restricted to this group. Effectors from the CRN family are in contrast ubiquitous and have been found in all examined oomycetes (Bozkurt *et al*., 2012). The RXLR amino acid motif present in the N-terminal region of such proteins is very similar to a signal for host targeting of malaria parasites (*Plasmodium* spp.), which suggests that this sequence might be highly conserved in different kind of pathogens (Birch *et al*., 2006), hence supporting the assumption of its essential role on the translocation into the host cytoplasm (Morgan & Kamoun 2007, Schornack *et al*., 2009). Up to now, all characterized avirulent proteins possess this host

translocation domain characterized by its RXLR motif (Arg-X-Leu-Arg) which confirms its importance in the infection process (Chen *et al.,* 2014). The fact that these effectors interact with intracellular host proteins, causing a hypersensitive reaction gave birth to the idea that these Avr proteins should be internalized in the plant cell (Morgan & Kamoun 2007). Several works confirmed that many RXLR and CRN effectors possess nuclear localization signals (NLS) which verifies their role in manipulating gene expression to confer the pathogen a higher virulence (Liu & Coaker 2008, Schornack *et al.*, 2010, Caillaud *et al.*, 2012, Mafurah *et al.*, 2015). Transient expression studies have demonstrated that RXLR effectors can exert their activity in divergent plant species, suppressing the immune response, which also confirms that the incorporation of these effectors in the cell happens through host mechanisms (Anderson *et al.*, 2012, Yin *et al.*, 2015). Numerous studies on oomycetes effectors have been conducted, targeting the discovery of new pathogenic molecules, its traffic inside host cells and its biochemical functions. This has been extensively reviewed (Kamoun 2006, Govers & Bouwmeester 2008, Hogenhout *et al.*, 2009, Schornack *et al.*, 2009, de Jonge *et al.*, 2011, Bozkurt *et al.*, 2012, Fawke *et al.*, 2015, Oliveira-Garcia & Valent 2015).

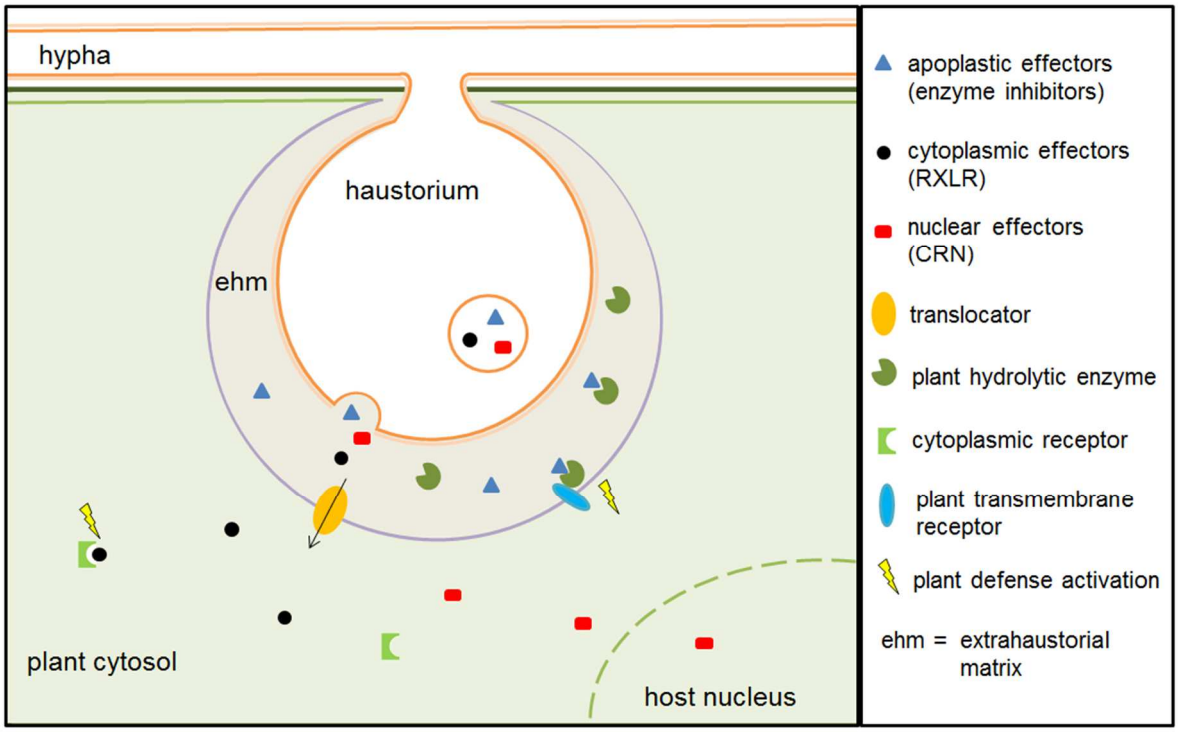

Figure 4.1. Interaction between a biotrophic pathogen and its host. A specialized infection structure (haustorium) penetrates the host cell wall and releases effector molecules. Effectors might be recognized by the plant activating a defense reaction.

Even though progress has been made in the breeding of resistance against downy mildew, the responsible mechanisms have not been elucidated. A very broad range of reactions were found in chapter 1 and 2 when strains of the pathogen were tested on hosts with different resistance levels. The established system allowed the selection of five strains characterized by showing different infection reactions. The molecular characterization of these strains performed in chapter 3 revealed differences at the genetic level. In the present chapter, attempts were made to unravel the reasons for the different pathogenicity. The expression of several putative effector genes, believed to play a role in the pathogenicity of the oomycete, was studied at different developmental stages.

At the beginning of our research, a very limited number (25) of putative effectors were known from *P. viticola* (Mestre *et al*., 2012), compared to *Phytophthora infestans* for which 563 genes are predicted to function as effectors (Haas *et al*., 2009). Our task was to confirm the presence of these effectors in the genome the five selected *P. viticola* strains, and compare their expression patterns. This goal was addressed during the master thesis of S. Becker (2015). The effectors studied in this chapter were discovered studying *in vitro* germinated zoospores (Mestre *et al*., 2012) generated by means of an already described protocol to induce zoospore encystment (Riemann *et al*., 2002). Our efforts to improve the single sporing technique for *P. viticola* during the bachelor thesis of M. Fröhler (2012) and N. Bendel (2013), gave us new tools to improve the *in vitro* encystment and germination of spores using different salts.

The grapevine cultivar Regent was selected based on its importance for viticulture and its high tolerance against downy mildew. This variety bred in the institute for Grapevine Breeding Geilweilerhof and released in 1996 has several North American wild species in its background which potentially contributed with resistance genes (Fischer *et al*., 2004). As this cultivar is not totally able to avoid being infected by *P. viticola*, its resistance might be classified as a 'horizontal' or quantitative resistance and is characterized by reduced development and sporulation of the pathogen. This kind of resistance, in the long term, is expected to be more durable than qualitative resistances which might be overcome by a single mutation (Pariaud *et al*., 2009). Fluorescence microscopy carried out by M. Kaiser (2015) during his bachelor thesis, followed the infection process of two strains on this tolerant cultivar. These observations confirmed the previously shown difference in virulence of the two strains, and encouraged the

study of effector gene expression in this host-pathogen combination. In a time in which large sets of oomycetes effectors are being discovered (Derevnina *et al*., 2015) and more *P. viticola* effectors enter the scene (Li *et al*., 2015, Yin *et al*., 2015), the identification of those decisive genes for a compatible infection is a key point in the molecular biology of plant pathogen interaction.

4.2 Material and methods

4.2.1 Developmental stadia

To compare the gene expression of the putative effectors in the selected strains, different host-independent and -dependent developmental stages of *P. viticola* were selected. The comparison of the five strains was performed using germinated spores (early stage) and infected leaf discs 96 hours post inoculation (late stage) (Fig. 4.2). For the host-pathogen combination study, infected leaf discs 6, 12, 24 and 96 hours post inoculation were studied

Figure 4.2: *Plasmopara viticola* **a**=sporangia, **b**=germinated spores and **c**=infected leaf disc 96 hours post inoculation used for the gene expression study of the five strains.

4.2.2 *In vitro* encystment and germination of spores

To be able to study the gene expression in a very early, host independent, but physiologically active stadium of the pathogen, it was taken advantage of the ability of zoospores to encyst and germinate via germ tube. Although zoospores naturally undergo this process just when they are located in a water layer covering the leaf surface, this process is individual and not synchronized in the zoospore population. To obtain a uniform sample condition, chemical stimuli were tested *in vitro* to induce encystment and germination of spores in a host-free system.

4.2.2.1 Mannitol and sorbitol

To determine the effect of the organic substances on the development of zoospores, strains 1136-A15 and 1137-C20 were selected. These experiments were conducted in the course of the B.Sc thesis of M. Fröhler (2012). Sporangia suspensions were prepared following the protocol described in 1.2.2. Square Petri dishes with 25 wells were used

and in each well 500 µl of the sporangia solution were dispensed. Concentrations of 10, 50, 100 and 200 mM mannitol and sorbitol were tested. Five wells were adjusted to each of the evaluated conditions and five wells were used as control. After an incubation of 24 hours in the darkness at 18 °C, the frequency of different stages (empty sporangia/full sporangia and encysted spores/destroyed spores was scored by means of an inverted microscope.

4.2.2.2 Sodium chloride and calcium chloride

Preliminary tests of Fröhler (2012) had shown that the addition of inorganic salts to sporangia solutions negatively affected the release of zoospores. Therefore, sporangia solutions were prepared as previously described and the salt concentration was adjusted after zoospores releasement (1-2 hours). These experiments were conducted in the course of the B.Sc thesis of N. Bendel (2013).

Three *P. viticola* single sporangium strains from different field isolates (1135-F15, 1137-C20 and 1191-B11) were selected to test the effect of salts addition after zoospore releasement. Strains were subcultured and handled as described in section 1.2.1. Multitest-3 wells glass slides (Menzel Glässer, Braunschweig) were used for this purpose. Sporangia suspensions (6 x 10^5 sporangia/ml) were prepared as in section 1.2.2 and distributed in the multitest slides. After 2,5 hours incubation in darkness at 18°C in a wet chamber, an abundant releasement of zoospores was achieved and the salt concentration was adjusted (50 µl final volume). Sodium chloride and calcium chloride were tested in concentrations of 5, 10, 15, 20 and 25 mM. The frequency of different stages (zoospores, encysted and germinated spores) in 100 counted spores was scored directly on the multitest slide 30 and 60 minutes after the addition of salts using a light microscope. This methodology was based on a previously described methodology of Riemann *et al*., (2002) and the experiences personally made during the supervision of the B.Sc thesis of Fröhler (2012). Two independent experiments were performed for each salt concentration and strain, and three technical replicates were scored in each experiment. In case of strong divergence, assays were repeated. Statistical analysis was conducted using InfoStat vers. 2013 (InfoStat Group, Argentina). By means of an Axioplan microscope (Zeiss, Oberkochen), pictures of the germinated spores were taken one hour after the salt addition, stained with Blankophor® (Blankophor GmbH & Co. KG, Leverkusen, Germany). The pictures were taken using a digital camera (Canon Power

Shot A640). A fluorescence filter (Zeiss, filter II, 02 /G365, excitation: 365 nm) was employed under UV light. To examine the effect of the addition of $CaCl_2$ in the following stages of the *P. viticola* infection, a leaf disc test was performed as described in section 1.2.2. Released zoospores treated with 20 mM calcium chloride were used to inoculate five leaf discs in concentrations of 100, 500, 1000, 5000 and 10000 sporangia per leaf disc. A water control was performed in parallel. This experiment was performed two times independently.

The reaction to calcium chloride was further investigated in other oomycetes using sporangia from *Plasmopara halstedii* (isolate 1211), *Pustula helianthicola* (isolate 1236) and *Pseudoperonospora humuli* (field isolate, botanical garden, University of Hohenheim). Trials were performed as previously described using concentrations of 10, 25 and 50 mM. Zoospore development after 30, 60, 90 and 120 min was observed and the frequency of each stage was scored. Experiments were performed in triplicate. Pictures from the stage of 120 min after the salt addition were taken under a light microscope.

4.2.3 Germinated spores

The induction of zoospores release was performed in 50 ml Falcon tubes filled with 3 ml of distilled water. To gain the sporangia, an infected leaf fully covered with sporangia of the selected strain was placed into the tube and strongly shaken. After removal of the leaf, the sporangia suspension was kept in darkness at 18°C for two hours. Samples were taken every 30 min to check the release of zoospores under the light microscope. When a strong release of zoospores was detected, 3 ml of a 100 mM $CaCl_2$ solution was added to the tube to give a final concentration of 50 mM. This concentration was chosen based on experiences gained in the previous experiments to induce encystment and germination of spores. Following this step, the suspension was incubated for another two hours under the same conditions and checked regularly under the microscope. When the germ tube development of the spores was detected, 2 ml of the suspension was added in a 2 ml reaction tube and centrifuged at 4 °C for two minutes at 15000 rpm. Afterwards, the supernatant was carefully discarded and the rest of the suspension was added to the reaction tube. A second centrifugation was performed. This step was repeated a third time until the whole suspension was centrifuged and a concentrated pellet of germinated spores was obtained. The tubes were shock-frozen on liquid

nitrogen and kept until use at -70°C. These experiments were conducted in the course of the M.Sc thesis of S. Becker (2015).

4.2.4 Putative effectors

The presence of the 25 known (at the date of the experiments) putative effectors from *P. viticola* (Mestre *et al*., 2012) was studied in the five selected strains. The nucleotide partial sequences of the putative effectors were downloaded from an EST library available online in the GenBank®, National Center for Biotechnological Information (NCBI). Specific primers were designed to amplify the desired regions using the software module Primer Select from Lasergene (V7.0.0, DNASTAR, inc., Madison, USA). PCR reactions were performed under the conditions mentioned in Table 4.1. Primers and its specific PCR product size are listed in Table 4.2. To corroborate the specificity of the designed primers, selected PCR products were sequenced (see section 3.2.2) and compared with the known effectors sequences using the Basic Local Alignment Search Tool (BLAST). These experiments were conducted in the course of the M.Sc thesis of S. Becker (2015).

Table 4.1: PCR conditions used for the analysis of putative effector genes (Mestre *et al*., 2012).

Step	Temperature	Time
Initial denaturation	94°C	2 min
Denaturation	94°C	10 sec
Annealing	60°C	10 sec
Elongation	72°C	10 sec
Final elongation	72°C	2 min

Table 4.2: Primers sequences and approximate size of the amplification products used to detect the presence of the putative effector genes in the genome of the *P. viticola* strains. Primers were designed in collaboration with Sandra Becker.

Primer name	Primer sequence 5´→ 3´	Expected fragment length (bp)
GIucInh_1_F	ACTCCCAACACCTCAGACGACA	726
GIucInh_1_R	TTCCGGTCACGAGTCCAATAAG	
GIucInh_2_F	TGGGGCTTCGAGGAGTGCTATT	300
GIucInh_2_R	CCGAAGACACGCGTGAGAAAAC	
GIucInh_3_F	ATAGCGCTGCCGATCAAGAGTA	378
GIucInh_3_R	CGTCGCAGCAAGGCAAGTTT	
GIucInh_4_F	ACGATGGGTGCTTTGCTGTATT	218
GIucInh_4_R	CCATGGGATAGCGACCGAC	
RXLR_1_F	CGCACTCAAAAGACAACACAAA	422
RXLR_1_R	GGTTAATAGCCCCGCAGACTT	
RXLR_2_F	CGGTGGCAAAGCAAGAGTTAC	671
RXLR_2_R	GCCGAAGACATTGGTGAGC	
ElicLike_1_F	GCTCCTCGTCGCATTTACATCT	512
ElicLike_1_R	GTCGAAGGGGTAGTGCTGTTTG	
ElicLike_2_F	TGCTGAGGCCACGGGTAT	565
ElicLike_2_R	GCTCGACGCACTGACACTGA	
1,3_Bgluc_1_F	GAGTCTCATGCTGCGGGTCAAC	558
1,3_Bgluc_1_R	GTCATGTGCACGAGCGGTAAGA	
1,3_Bgluc_2_F	CAAACTTTCCCGGCTCCTCA	616
1,3_Bgluc_2_R	TTCCAACTTTTCCACGCAATAA	
1,3_Bgluc_3_F	TTCAAGGCAAAGTCAATAAGTGGA	277
1,3_Bgluc_3_R	TTACTAGAAGACGGGAACAAGATGG	
1,4_Bgluc_1_F	CACGCGAAATTGTACTTGGATG	764
1,4_Bgluc_1_R	ACTGGATCGTTGGGGTCACA	
SecOP_1_F	CGAGCTCGACACGTTTGACTT	254
SecOP_1_R	GACGCAACAACTCGAAGGAACT	
SecOP_2_F	GAGGGTGGAGTAGGCTTCAATACA	306
SecOP_2_R	TGAAGGTTCTCACTCGGATGC	
PecEst_1_F	GCCGGTCCCGATTGTGAA	580
PecEst_1_R	GCCACGGCTTGTCCAGATTTT	
PecEst_2_F	GAGCAAGTCATCATCCCAAAAGA	680
PecEst_2_R	GTATTTGCACCAGGACCGTTG	
PecEst_3_F	CGTGTTGGTGCTGTTTTCATCA	416
PecEst_3_R	ACATTGTCGGTGGAGTCATTTTTG	
TransGlut_1_F	CGGGTTTCTTCCACATTGCTAC	732
TransGlut_1_R	CGGCGGCTCGGTATTTTC	
TransGlut_2_F	AACCCCTGCATTCTACCATCTT	390
TransGlut_2_R	CTCTCCAGCGTCATTCAACTCC	
AcidChit_F	GACAGCCACCAACCATTCAAA	731
AcidChit_R	ACCGCACTCGAGTCCACCAT	
CysProt_1_F	GAAACGATGGATGTGCGAACTC	850
CysProt_1_R	GATGCCGAGGTTATTGTGATGC	
CysProt_2_F	CAAGGGCAAATGTGGTTCGT	631
CysProt_2_R	GGCCAACCAGCATTCAAGATT	
CystatinLike_1_F	TCGACTGGGAGCGTGTTCA	136
CystatinLike_1_R	TTCTCCCACGTCAACTACTCAGG	
CystatinLike_2_F	GCTACGCGGCAAGTGACG	222
CystatinLike_2_R	TTCTCCCACGTCAACTACTCAGG	
KazalLike_F	GAAGACGCCGAACATCCAGA	330
KazalLike_R	GCGACACGATTACAAGCAAGAA	

4.2.5 Leaf disc infections

Grapevine (*Vitis vinifera* cv. Müller-Thurgau) plants were grown in a greenhouse at 25°C. Leaves from the fourth to the seventh position from the apex were detached and rinsed with distilled water. Leaves from two plants were used in order to compensate individual reactions. Leaf discs of 1 cm diameter were excised using a cork borer. The discs were placed abaxial side up on Petri dishes in which the bottom was covered with filter paper soaked with distilled water (Fig. 4. 3). Ten leaf discs were prepared for each strain and each time point.

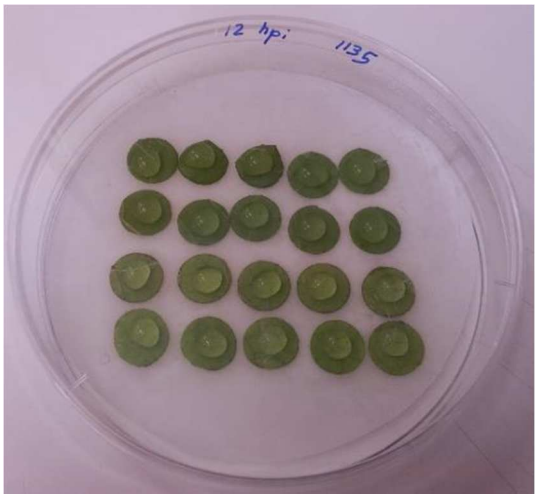

Figure 4.3. Leaf discs inoculation system. Droplets of 40 µl inoculum were placed on the abaxial leaf surface.

The inoculum was obtained from profusely infected leaves. Leaves were rinsed two days before and left in the growing chamber to ensure fresh sporangia production. Falcon tubes (50 ml) containing 2 ml of distilled water were used to collect the sporangia from the infected leaves by shaking them inside the tube. The suspension of sporangia was adjusted to 50 000 sporangia/ml (Fuchs-Rosenthal counting chamber).

Leaf discs were inoculated with 40 µl of the sporangia suspension (see Fig. 4.3) and kept in a climate chamber at 18°C , the first 24 h in darkness and then under a 14 h photoperiod. Drops were removed from the surface after 48 hours to avoid secondary infections by other microorganisms. After 96 hours, five leaf discs were collected in a 2 ml reaction tube, shock-frozen with liquid nitrogen and kept at -70°C. Positive controls (five inoculated leaf discs) were kept in the growing chamber for six more days to

evaluate the infection. Some of these experiments were conducted in the course of the M.Sc thesis of S. Becker (2015).

4.2.6 RNA isolation from sporangia and germinated spores

RNA was extracted from sporangia and germinated spores using the Aurum ™ Total RNA Isolation Kit (Bio-Rad Laboratories GmbH, Munich). To the reaction tubes containing the frozen material, three stainless steel grinding balls (2,8 mm, Precellys, Peqlab, Erlangen) and 500 µl cold lysis solution from the Kit were given. The samples were then placed in a mixer mill (MM300, Retsch GmbH, Hann) for 2 min at a frequency of 30 Hz. Next steps were performed according to the manufacturer instructions. Subsequently, the contamination of the RNA samples with genomic DNA was controlled by means of PCR using *P. viticola Actin* primers (Table 4.3). Samples contaminated with genomic DNA were digested using DNAse I (PerfeCTa® DNase I, Quanta Biosciences, Gaithersburg, USA) according to the manufacturer's instructions. Some of these experiments were conducted in the course of the M.Sc thesis of S. Becker (2015).

4.2.7 Total RNA isolation from infected leaf discs

The RNA extraction of the infected plant material was conducted using the GeneMATRIX Universal RNA Purification Kit (Roboklon GmbH, Berlin). Three stainless steel grinding balls (2,8 mm, Precellys, Peqlab, Erlangen) were added to the reaction tubes containing the frozen material and the samples were grinded three times at 30 Hz for 30 seconds using a mixer mill (MM300, Retsch GmbH, Hann). Between each grinding cycle, the samples were frozen in liquid nitrogen to keep them cold and avoid RNA degradation. Next steps were performed according to the manufacturer instructions. Samples contaminated with genomic DNA were handled as in 4.2.6. The RNA concentration in the samples was quantified using a NanoVue spectrophotometer (GE Healthcare, Waukesha, Wisconsin). Some of these experiments were conducted in the course of the M.Sc thesis of S. Becker (2015).

4.2.8 cDNA synthesis

Right after the RNA extraction, reverse transcription (RT) of the RNA was performed to avoid degradation. From each sample, the same amount of RNA was used to assure similar conditions for the RT. The final concentration of the cDNA was 100 ng/µl. The

cDNA was synthesized starting from total RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot) according the manufacturer's instructions. The primer used for the first strand synthesis was the VNdT18-Oligonucleotide in a final concentration of 5 µM. Semi quantitative analysis of gene expression in the five strains was performed using capillary electrophoresis.

The concentration of cDNA in all the samples was adjusted considering the concentration of the amplified product in a first PCR experiment using specific primers for *P. viticola Actin*. This concentration is proportional to the peak area registered using microchip capillary electrophoresis (MultiNa). According to the peak area correspondent to each PCR product, the samples were adjusted to identical concentration before the semi quantitative experiments were performed.

4.2.9 Semi-quantitative analysis of gene expression in the five strains

For the semi-quantitative and quantitative analysis, short PCR products are preferred over larger products because their amplification efficiency is generally higher. For this reason, new primers were designed using the same sequences, but flanking regions shorter than 300 bp (Table 4.3). Primers were designed following the recommendations here listed: the size was kept between 18 and 28 bp. The temperature of melting (Tm) was set between 57 and 63 °C. The difference of the Tm of forward and reverse primers was not bigger than 1°C. The size of the amplified fragments was between 100-300 bp (most around 150 bp). Repeats of more than 3 Cs or Gs were avoided in the sequence. Primers ended in a G or a C residue. The GC content was kept between 40-60%. The specificity of the primers was checked using BLAST. HPLC purified primers were preferred.

To enable the comparison of the gene expression in the five *P. viticola* strains, the appropriate number of cycles in which the exponential phase of the PCR reaction is reached, was required. Reactions with different numbers of cycles (21, 24, 27, 30, 33. 36. 39) were performed using cDNA from one of the strains for the reference gene and one selected effector gene. PCR products were analyzed through microchip capillary electrophoresis and the peak area of each of them was assessed. The optimal cycle number was used for the further gene expression studies.

Table 4.3: Primers sequence used for the gene expression studies and approximate size of the expected PCR product. Primers were designed in collaboration with Sandra Becker.

Primer name	Sequence 5' → 3'	Approximate size (bp)
Elicitin like_1_RT_F	CGCTCCTCGTCGCATTTACATCTG	125
Elicitin like_1_RT_R	GCTCACGTTGGTCGCACACTCTTC	
Elicitin like_2_RT_F	CATGAGCAAGACTGGCAATGAGACTG	163
Elicitin like_2_RT_R	GACAGTGGAAGTGGAGGGAGACGC	
Gluc Inhib_2_RT_F	GGCAATTTCTCTAAAGAACTGCTGGCTATC	148
Gluc Inhib_2_RT_R	GTGGACCACCATTATCACCCTCGC	
Gluc Inhib_4_RT_F	CATAGACACGTGCGATTTTGATACTGG	153
Gluc Inhib_4_RT_R	CATGGGATAGCGACCGACACG	
Kazal like_RT_F	CTTTCTTGCTTGTAATCGTGTCGCTG	147
Kazal like_RT_R	GTCGCTTTGCTCGCTTCTCCTTAC	
RXLR_1_RT_F	CTCGGTGAAAAAGTTGTTGCTGGTG	125
RXLR_1_RT_R	CCGCTCGCGCTGTCTGAATC	
RXLR_2_RT_F	GGTACCCCCTTCTGCTGTTGTGC	134
RXLR_2_RT_R	GAATGTTCGCGTAATACCCAATCGTG	
1,3_ß Gluc_2_RT_F	CGTTGGACATAACACGACATTGCTTC	172
1,3_ß Gluc_2_RT_R	GTTGCCAAAATCTTCGTACAGTCCACC	
PvNLP1_F	TGATGCTTAAACCCGAACTTCAC	164
PvNLP1_R	CAAATGGTGCGTGTCGACCGTAAACTG	
P. vit. actin_2_RT_F	CTCACGTACATTGCCTTGGACTTTG	179
P. vit. actin_2_RT_R	GAATACCTGACGCTTCTTTACCAATGAG	
P.vit. ß tub_1_RT_F	CTTCAAGGTTTTCAAATTACGCACTCG	281
P.vit. ß tub_1_RT_R	CCGTACGTGGGAGTGGTGAGTTTC	
P.vit_EF1 α_F	GTTGCTTGCCTTTACGCTTGGAG	157
P.vit_EF1 α _R	CGGGATCTTCGCAGGCTTGTAG	

A PCR product with a known concentration was used to determine the linear measuring range of this methodology. Dilution series, paired with their detected peak area, were used to calculate the optimal working range of the MultiNa. Fluctuations between chips

were also taken into consideration. A PCR product (*RXLR 1*) of known concentration was analyzed repeated times using different chips in the MultiNa to assure reproducibility. Genomic DNA (10 ng) was used for this purpose. Some of these experiments were conducted in the course of the M.Sc thesis of S. Becker (2015).

The PCR protocol used in the gene expression study is described in Table 4.4. For the sporangia and infected leaves, 33 cycles were used and for the germinated spores 36 cycles. The analysis was restricted to three effector genes (*Elicitin like 1, Kazal like* and *RXLR 1*). Two independent experiments were performed for each stage of development and each strain. In each experiment, two technical repetitions were performed. The relative expression of the gene was calculated as the peak area of each gene of interest divided by the peak area of the reference gene *Actin*. The obtained value was then divided between the relative expression of the same gene in the sporangia (untreated sample) to get the relative normalized expression.

Table 4.4: PCR amplification reaction used for the semiquantitative expression study of the putative effectors using Red*Taq* Mastermix.

Step	Temperature	Time
Initial denaturation	94°C	2 min
Denaturation	94°C	10 sec
Annealing	60°C	10 sec
Elongation	72°C	10 sec
Final elongation	72°C	2 min

4.2.10 Quantitative analysis of gene expression in the five strains: real time PCR

The quantitative RT-PCR was performed with cDNA from the same synthesis reaction used in the semi quantitative study to assure comparable results. Two independent experiments were performed with three technical repetitions for each strain and stage of development. PCR conditions are listed in Table 4.5. The cDNA was amplified in a CFX96 Touch™ Real Time Detection System (Bio-Rad Laboratories GmbH, Munich). SYBR Green I was used for visualizing the amplification. SensiFast™ SYBR No-ROX Kit (Bioline, London, UK). The gene expression analysis was performed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories GmbH, Munich) for the same genes considered in section 4.2.9. The relative normalized expression ($2^{-\Delta\Delta CT}$) was calculated using sporangia cDNA as "untreated sample" and *Actin* as a reference gene.

Table 4.5: PCR amplification reaction used for the quantitative expression study of the putative effectors using SYBR Green I.

Step	Temperature	Time
Initial denaturation	95°C	2 min
Denaturation	95°C	15 sec
Annealing	60°C	15 sec

4.2.11 Host-pathogen combinations

4.2.11.1 Selection of isolates and cultivars

The fact that two *P. viticola* strains showed different degrees of infection on a tolerant *V. vinifera* cultivar shown in chapter one, motivated the analysis of the expression of effector genes on different time points of the infection. The strains 1135-F2 and 1137-C20 were selected, and a leaf disc test on the cultivars Müller-Thurgau and Regent was performed following the same procedure already mentioned. On the susceptible cultivar Müller-Thurgau, both strains achieved a profuse sporulation after seven days, while in the tolerant cultivar Regent, the strain 1137-C20 (highly virulent) showed an appreciably stronger sporulation compared with 1135-F2 (lowly virulent) (Fig. 4.4).

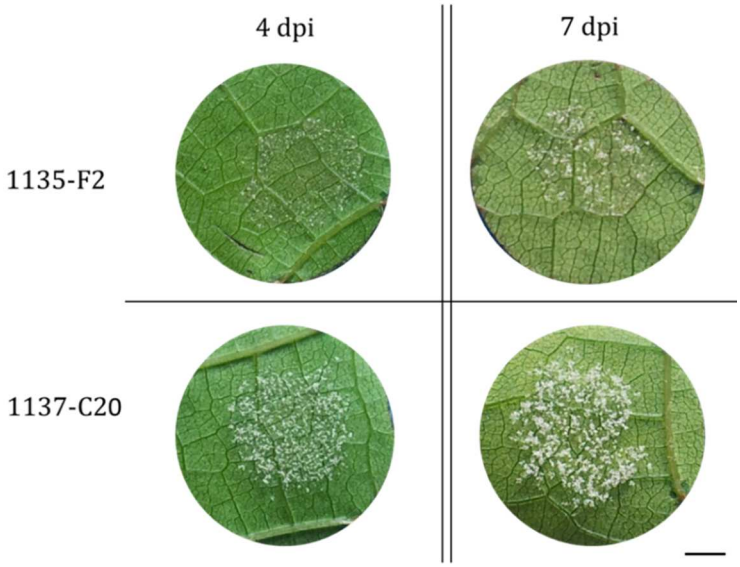

Figure 4.4: Leaf disc test on the tolerant *V. vinifera* cv. Regent, four and seven days after the inoculation with 5000 sporangia of the strains 1135-F2 or 1137-C20. Bar: 2 mm.

4.2.11.2 Leaf disc infection and microscopy

The leaf disc test was carried out as described above (4.2.5), but with the following modification: sporangia solutions were left for 2 h in darkness at 18°C to release the zoospores and the leaf discs were immediately inoculated applying the same conditions. For each host-pathogen combination and time point, 20 leaf discs were prepared. Five were left until the end of the experiment as a control for successful infection, five were fixed in FAA (formalin-acetic acid-alcohol) for microscopy, and ten were shock frozen on liquid nitrogen for the gene expression study. Leaf discs were collected after incubation periods of 6, 24 and 96 hours post inoculation. Three independent experiments were performed.

For microscopy analysis, leaf discs were clarified by heating them in 5% KOH at 95°C for 2-3 hours until the tissue was completely transparent. Aniline blue (0,05 %, 0,0067 M, K_2HPO_4, pH 9-9,5) was added to the discs and a short vacuum was applied to assure a good staining of the inner tissues. Following a distilled water rinse, leaf discs were placed on a glass slide and observed using fluorescence microscopy applying the same conditions mentioned in section 4.2.2.2. These analyses were performed by Markus Kaiser as part of his B.Sc thesis (2015).

4.2.11.3 Quantitative analysis of gene expression: real time PCR

Extraction of total RNA and subsequent cDNA synthesis were performed following the protocol already mentioned (4.2.6-4.2.8). PCR conditions listed in Table 4.5 were used to amplify the putative effector sequences for each host-pathogen combination and time point. The analysis was restricted to three effector genes previously studied with the five strain (*Elicitin like 1, Kazal like* and *RXLR 1*). Employed primers are listed in Table 4.3. The gene expression analysis was performed using the same procedures as in section 4.2.10.

Previous results had shown that 6 hpi was an important point in the infection and that at this point a very high upregulation of the putative effector genes occurred. Using this information, a screening was performed for the rest of known *P. viticola* effectors using the same cDNA. Those effectors which showed an upregulation at this point were selected for further experiments (Data not shown).

4.2.11.4 Effectors screening in the host-pathogen combination

Considering previous results, a new experiment was designed to screen for further interesting effector genes expressed during the interaction of a virulent strain with a tolerant cultivar. In this experiment, the focus was placed on earlier infection points (0, 6, 12 and 24 hpi). The leaf-disc infection was performed as previously described (4.2.11.2) with some modifications: each sample consisted of 10 discs 5 mm in diameter and excised from the inoculated discs (a disc from the disc). This was done in order to avoid any edge effect in the host reaction due to wounding. The samples were collected at each time point on 2 ml reaction tubes and shock-frozen using liquid nitrogen. Inoculated leaf-discs were left for five days as a sporulation control in all the host-pathogen combinations. Incubation was performed under the previously described conditions. Experiments were repeated, if the control discs did not sporulate.

Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) coupled with a DNA digestion performed using the RNase-Free DNase Set (Quiagen) according to the manufacturer´s instructions. The new RNA isolation kit allowed us to obtain much higher concentration of RNA. This was necessary to screen many genes, especially those with a lower expression. The quality and concentration of the RNA was controlled using a BioPhotometer (Fa. Eppendorf, Hamburg). The cDNA synthesis was carried out as previously described (4.2.8).

Quantitative RT-PCR was performed as described in point 4.2.9 for the six selected genes (*RXLR 1, NLP 1, Elicitin like 2, Glucanase inhibitor 2, Glucanase inhibitor 4* and *1,3 ß Glucanase 2*) (see Table 4.3). Primers for *NLP 1* were obtained from Dipl.-Biol. Stefan Schumacher from the Institute of Enology and Viticulture in Freiburg (WBI) as part of a cooperation framework. Three independent biological replicates were performed with three technical repetitions each. The gene expression analysis was performed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories GmbH, Munich). The relative normalized expression ($2^{-\Delta\Delta CT}$) was calculated using the inoculated leaves 0 hpi as "untreated sample" and three reference genes (*Actin, Tubulin* and *Elongation factor 1 α*) were used for normalization.

Another goal was to get information about the reaction of the plant during the first infection stages. Using the same samples, the expression of defense related genes from

the plant (S*tilbenes synthase, Metacaspase 2* and *5* and *NBS-LRR 2*) was analyzed by M.Sc. Peijie Gong from the Karlsruhe Institute of Technology as part of his doctoral thesis. Some of his results were used for discussion.

4.3 Results

4.3.1 *In vitro* encystment and germination of zoospores

4.3.1.1 Mannitol and sorbitol

In all the concentrations tested, sorbitol and mannitol showed a negative impact on the zoospore release from the sporangia. Zoospore encystment after 24 hours was lower than 5 % using both substances and in both tested strains (for detailed data see B.Sc thesis of M. Fröhler, 2012). Therefore, no longer testing was performed using organic substances.

4.3.1.2 Sodium chloride and calcium chloride

In order to avoid negative effects on the zoospore release due to osmotic stress (Fröhler 2012, Bendel 2013), the influence of sodium and calcium concentration on zoospores were tested only after the release from sporangia had occurred (see 4.2.2.2). Already 30 min after salt application, numerous encysted spores were found, especially in the higher concentrations (15-25 mM). After 60 min, already a high number of germinated spores were recorded, much higher than in the first 30 min. Because the biggest differences between salts and concentrations were found when germinated spores were used as indicator, subsequent statistical and graphical analyses were performed based on this value. The behavior of the three strains 1135-F15, 1137-C20 and 1191-B11 tested in this experiment was appreciably different when considering the salt concentration and the time point in which they were evaluated. This indicated a genotype depending reaction.

After one hour, more spores developed a germ tube when $CaCl_2$ was added compared to NaCl in the concentrations of 15, 20 and 25 mM. This result was particularly conclusive for the isolates 1137-C20 and 1191-B11. Isolate 1135-F15 behaved interestingly different, showing a better response to NaCl and achieving much higher frequency of germinated spores when this salt was applied (Fig. 4.5).

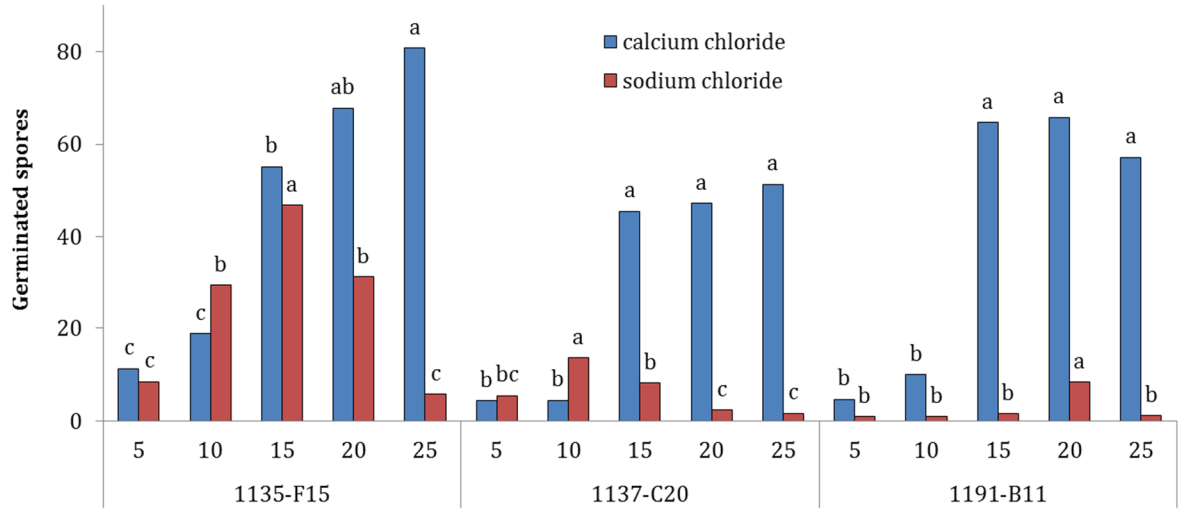

Figure 4.5: Relative frequency of germinated spores of the three *P. viticola* strains evaluated 60 minutes after the addition of NaCl and $CaCl_2$ to a final concentration of 5, 10, 15, 20 and 25 mM. Different letters indicate significant differences ($p<0,05$; independently for each isolate and salt).

Calcium chloride in concentrations of 15, 20 and 25 mM promoted a quick development of the zoospores, from their motile state, to the encystment and germ tube formation. No difference was found in the frequency of germinated spores (Fig. 4.5), or in the development of the spores (Fig. 4.6) using these three concentrations. In all cases, a long germ tube was produced after 60 minutes, which in some cases presented a branching growth.

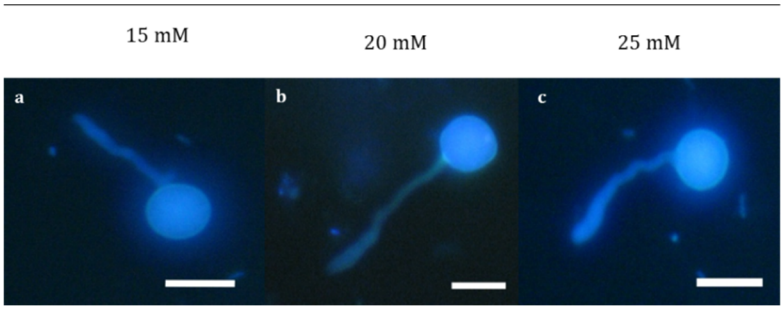

Figure 4.6: Fluorescence microscopy image 60 minutes after the addition of $CaCl_2$ of germinated spores belonging to the strain 1137-C20 stained with Blankophor®. Bar: 10 µm. Pictures were taken by Nele Bendel.

Due to the satisfactory results obtained using $CaCl_2$ for the *in vitro* encystment and germination of the spores, the mean concentration (20 mM) of this salt was selected for the subsequent experiments. A leaf disc test, in which a zoospore suspension was treated with calcium chloride (20 mM final concentration), was performed on the susceptible cultivar Müller-Thurgau. This test showed that the infection strength was

higher, compared to the water control in all tested sporangia concentrations (Fig. 4.7). This was an indicator that the addition of salt in this concentration positively affected the development and infection of *P. viticola*. Single zoospore infections were attempted using germinated and encysted spores without success.

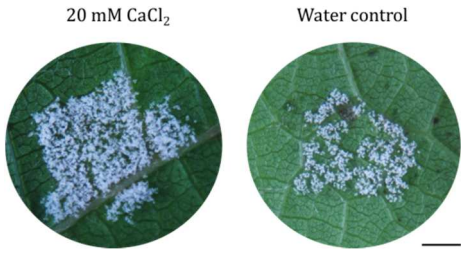

Figure 4.7: Effect of treatment with 20 mM $CaCl_2$ on the *P. viticola* infection process. Left, sporangia solution supplied with $CaCl_2$. Right, sporangia solution in water. Evaluation 10 days post inoculation with 10000 sporangia per leaf disc. Bar: 1 mm. Pictures were taken by Nele Bendel.

4.3.1.3 Salt effect on encystment and germination of spores on other oomycetes

In contrast to the water control, 90% of the released *P. halstedii* zoospores had already encysted only 30 min after application of $CaCl_2$ in the three tested concentrations. This result differs from that obtained in the other two oomycetes, where a high ratio of encysted spores was not found until 90 min after treatment. Nevertheless, 120 min after the salt addition, the three studied oomycetes showed more than 40% of germinated spores, partially with very long germ tubes (Fig. 4.8). The $CaCl_2$ concentration in which the highest number of germinated spores was scored was 25 mM. However, at 50 mM the encystment and germination of the spores was faster.

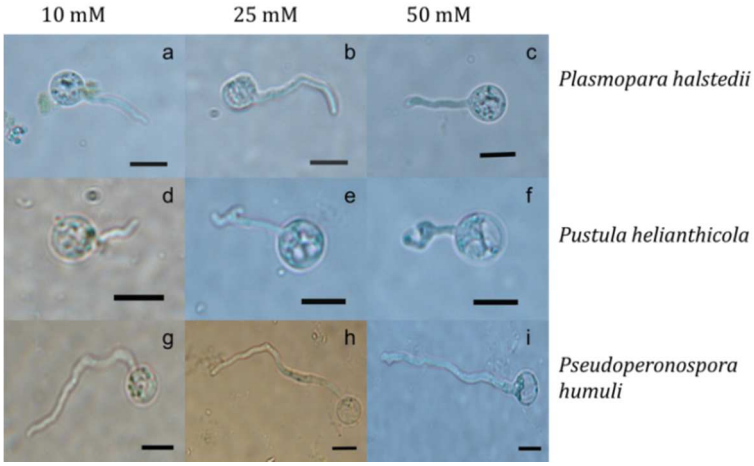

Figure 4.8: Light microscopy of germinated spores from the three studied oomycetes 120 minutes after the addition of $CaCl_2$. Bar: 10 µM. Pictures were taken by Nele Bendel.

4.3.2 Putative effectors

The presence in the genome of the 25 effector genes (Mestre *et al*., 2012) was confirmed for the five selected *P. viticola* strains using PCR and agarose gel electrophoresis (data not shown). In some cases where no PCR product was detected in any of the strains, the design of new primers was necessary. The sequences of the primers listed in Table 4.2 are those which successfully amplified the desired regions. The predicted size of the PCR product given by Mestre *et al*., 2012 was in some cases slightly different to the obtained product size. Nevertheless, BLAST analysis with the reported sequences allowed us to confirm that the amplified regions corresponded to the selected effector.

4.3.3 Semi quantitative analysis of gene expression in the five *Plasmopara viticola* strains: capillary electrophoresis.

The gene expression in all five strains was very similar and all of them showed the same reaction pattern (Becker 2015). While *Elicitin like 1* and *Kazal like* were not upregulated, *RXLR 1* showed a very high level of upregulation (Fig. 4.9). Germinated spores in contrast with infected leaves 96 hpi presented a very high content of the *RXLR 1* transcript in the five strains. Due to a high variability and a low number of samples, it was not possible to determine if the difference between them was statistically significant. *Elicitin like 1* and *Kazal like* were expressed in all analyzed stages, but their expression was very constant in relation to *Actin* and behaved very similar in the sporangia, germinated spores and infected leaves.

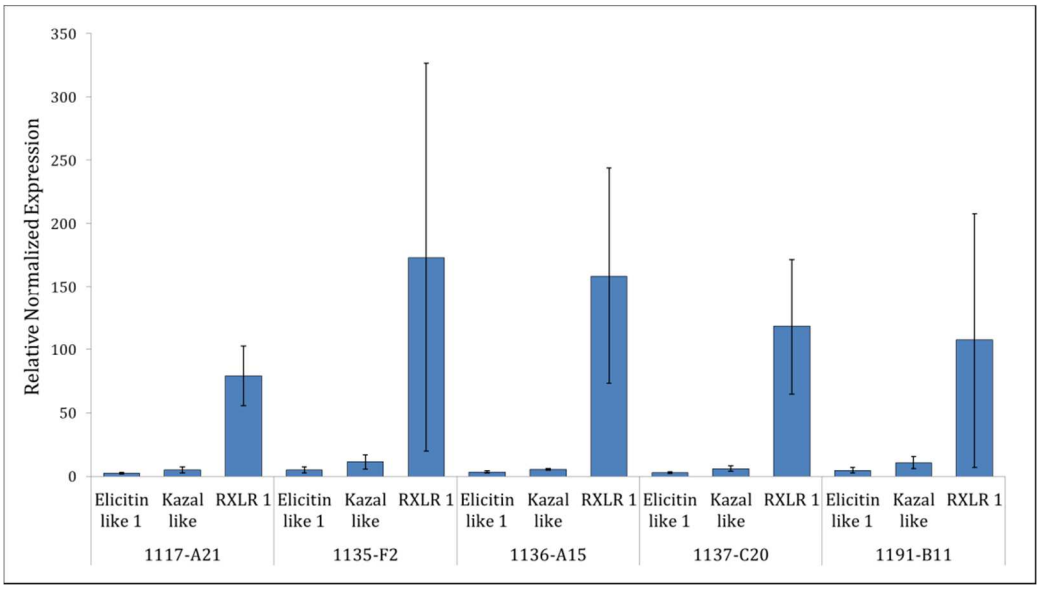

Figure 4.9: Relative normalized expression (semi-quantitative) of three putative effector genes in germinated spores of five selected *P. viticola* strains. The gene expression in sporangia was used to normalize the results and *Actin* as a reference gene.

4.3.4 Quantitative analysis of gene expression in the five strains: real time PCR.

Quantitative real time PCR experiments showed similar results compared to the semiquantitative method. *Elicitin like 1* and *Kazal like* remained unregulated while *RXLR 1* presented again a high upregulation in the germinated spores. The stadium 96 hpi showed no upregulation of this gene, either (Fig. 4.10). A high variability was also found in the expression of *RXLR 1* in germinated spores, showing how sensitive the system is. The induction fold of the *RXLR 1* was much higher (up to 1700-fold, 1137-C20) in the quantitative compared to the semi-quantitative method (120-fold), but the trend was similar.

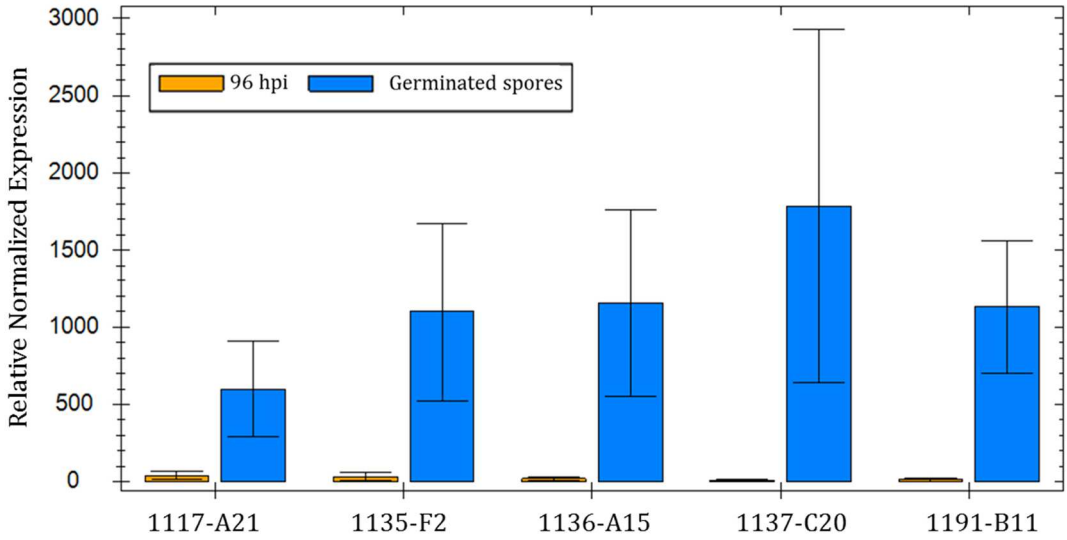

Figure 4.10: Relative expression of *RXLR 1* in germinated spores and leaf discs 96 hpi of five selected *P. viticola* strains. The gene expression in sporangia was used for normalization and *Actin* as a reference gene.

4.3.5 Infection development on different cultivars

In the first hours of the infection it was possible to observe how the zoospores targeted the stomata. After encysting, spores germinated, penetrated the substomatal cavity and built a germ tube. Contrary to the expectation, no difference was found between the infection process of the two selected strains 1137-C20 and 1135-F2 at 6 and 24 hpi. The virulence difference between the strains in previous infection experiments (Fig. 2.2) was not evident until 48 hpi, where a more dense coverage of mycelia was appreciable in the leaf discs of Regent (tolerant cultivar) infected with 1137-C20 (highly virulent). In the susceptible cultivar Müller-Thurgau, no differences in the development of mycelia were detected between the two strains along the whole infection process. While strain 1137-C20 in Regent at 96 hpi covered a broad leaf area, strain 1135-F2 presented much narrower leaf colonization. The highly virulent strain achieved sporulation at 72 hpi in Regent in contrast with the lowly virulent strain, where only sparse sporulation was found after 96 hpi, indicating a slower growth in the tolerant cultivar (Fig. 4.11).

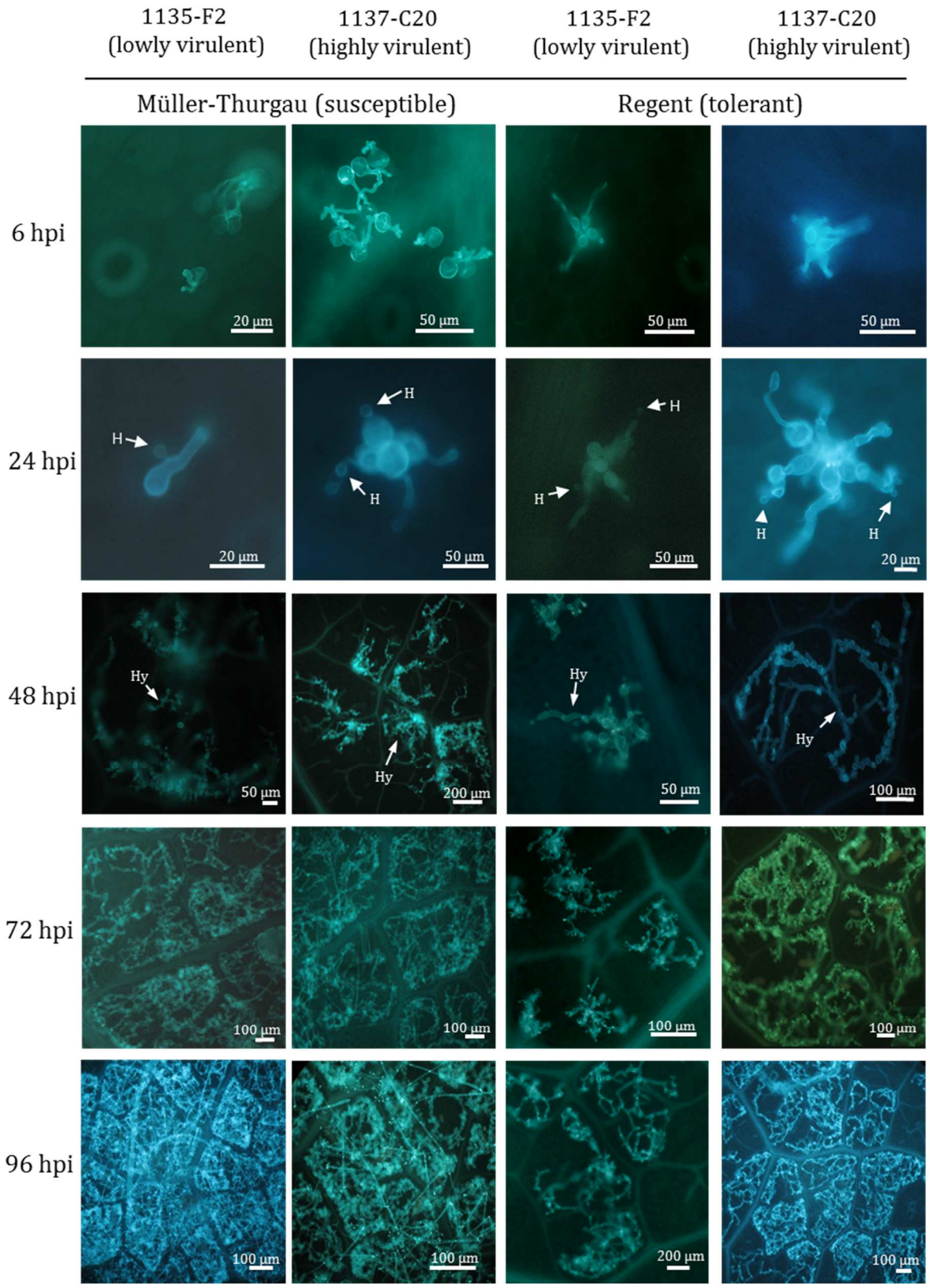

Figure 4.11: Infected leaves of a susceptible (Müller-Thurgau) and a tolerant (Regent) grapevine cultivar. Strains 1135-F2 (low-virulent) and 1137-C20 (high-virulent) were inoculated. Samples were evaluated 6, 24, 48, 72 and 96 hpi using fluorescence microscopy. H=haustorium, Hy=hypha. Aniline blue was used for staining. Pictures were taken by Markus Kaiser.

4.3.6 Gene expression of selected strains on different cultivars

The three studied effectors showed very similar results in the host-pathogen combination study as in the early experiments. *Elicitin like 1* and *Kazal like* were not upregulated in none of the analyzed time points of the infection. Just *RXLR 1* experienced an upregulation with a peak at 24 hpi in the lowly virulent strain (1135-F2) and at 6 hpi in the highly virulent strain (1137-C20). The expression level at 96 hpi was in all the cases the lowest found for this effector. In both cases the expression level of *RXLR 1* was higher when the strains were inoculated on the tolerant cultivar compared with the susceptible one (Fig. 4.12).

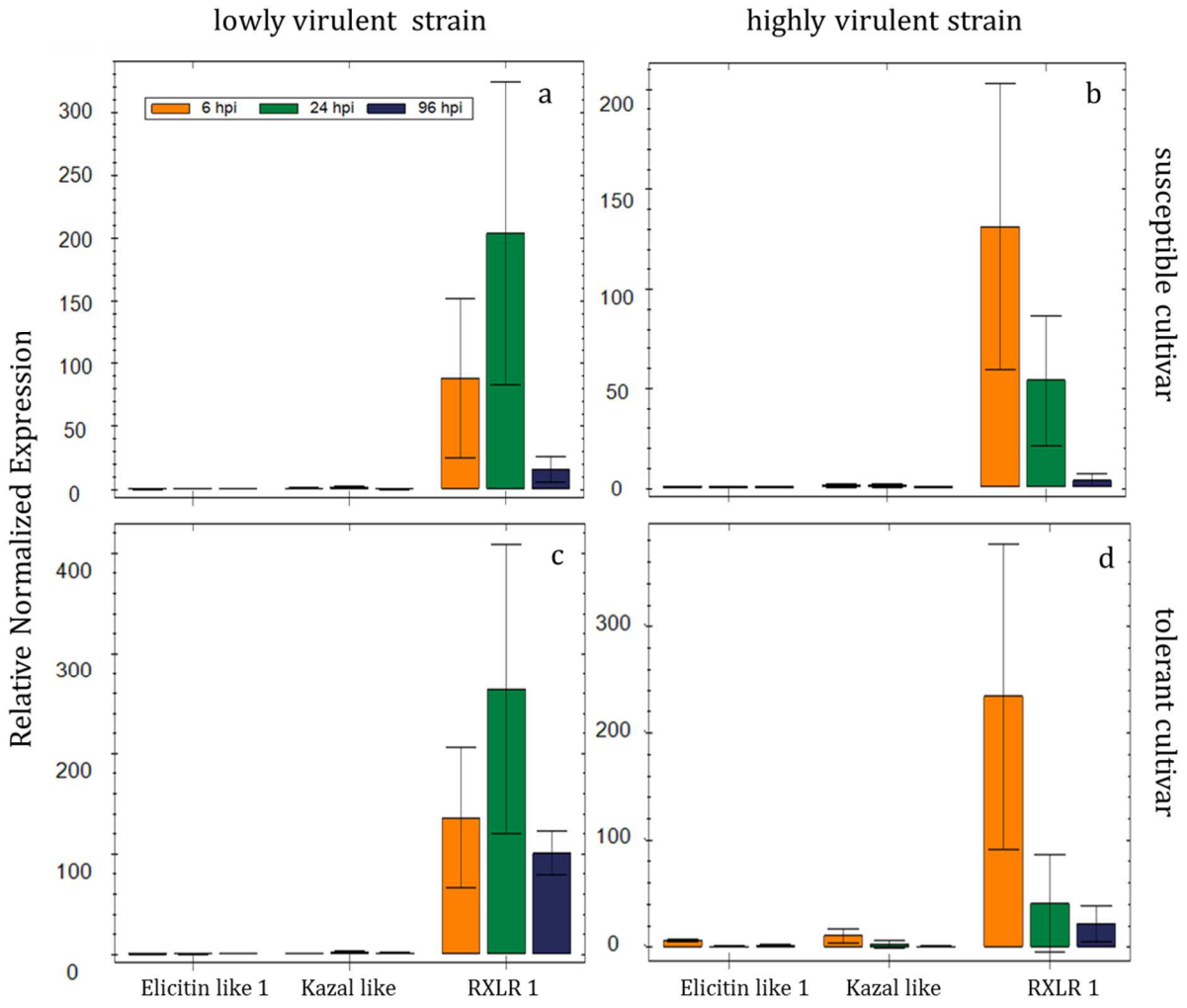

Figure 4.12: Relative normalized expression of the three putative effector genes (*Elicitin like 1*, *Kazal like*, and *RXLR 1*) of the strain 1135-F2 (lowly virulent) and 1137-C20 (highly virulent) infecting the susceptible (Müller-Thurgau) and tolerant (Regent) cultivars at 6, 24 and 96 hours post inoculation. Presented are means of two independent experiments. Error bars represent the SEM.

The lowly virulent strain presented the same pattern in both cultivars. At 6 hpi the level of expression was already high, but increased at 24 hpi before a decrease at 96 hpi. In the tolerant cultivar the level of expression decreased less pronounced than the susceptible one at 96 hpi. The highly virulent strain showed a different pattern with the highest expression at 6 hpi followed by a decrease in the further time points.

4.3.7 Effectors screening in the host-pathogen combination Müller-Thurgau and Regent with 1137-C20 and 1135-F2

Five out of the twenty eight studied effectors, beside *RXLR 1*, showed interesting results and the complete analysis was performed using those selected genes. *RXLR 1* showed the highest upregulation of all the tested genes (Fig. 4.13). In the susceptible cultivar the highly virulent strain (1137-C20) showed a higher expression level than the lowly virulent strain (1135-F2) at 6 hpi, similar to the results from the previous section. At 12 and 24 hpi, the expression levels strongly decreased, in contrast to the previously observed increment in the expression with a peak at 24 hpi. In the tolerant cultivar the same expression pattern was found, but in this case the expression was 3-fold higher at 6 hpi. After 12 and 24 hpi the expression was still high, but considerably lower than at 6 hpi, contrasting again with the previous results.

A similar pattern presented *NLP 1* where the strain 1137-C20 showed a very high level of expression at 6 hpi in the tolerant cultivar (7-fold higher than in the susceptible). In the susceptible cultivar the expression in both strains was very low and just slightly higher at 6 hpi (Fig. 4.14). *RXLR 1* and *NLP 1* were the only genes for which the highest expression was found in the highly virulent strain infection on the tolerant cultivar. This suggests an important role of these genes in the infection process of a tolerant cultivar.

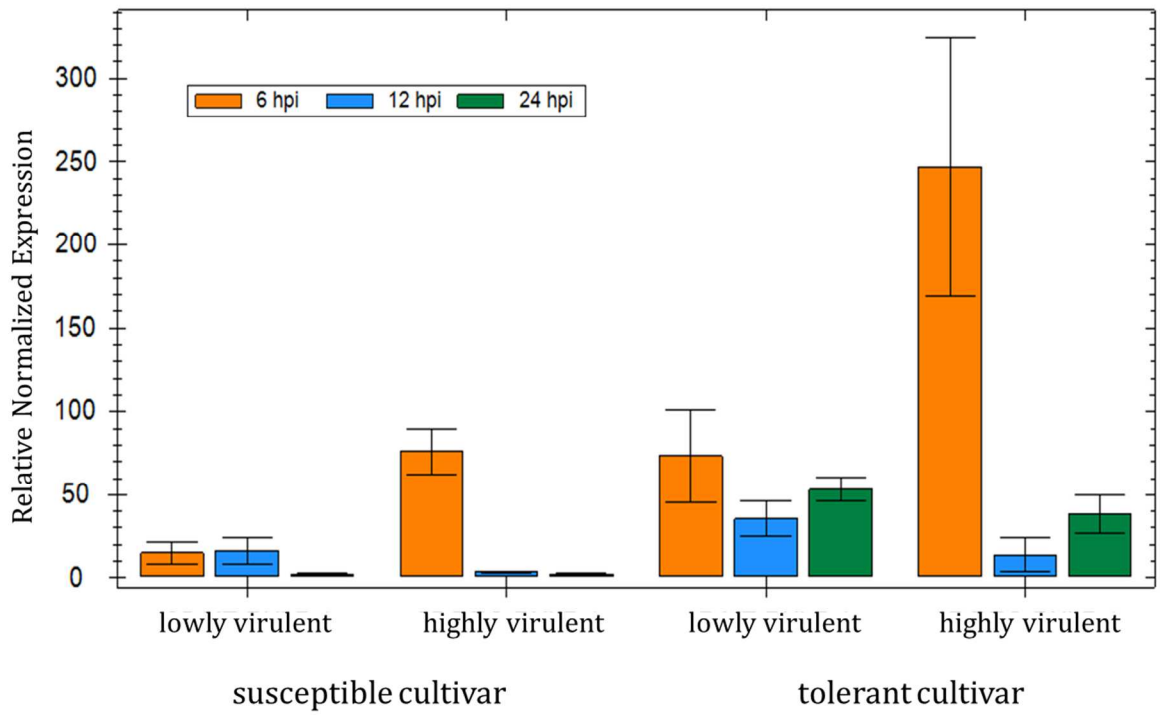

Figure 4.13: Relative normalized expression of the putative effector gene ***RXLR 1*** on the lowly virulent strain (1135-F2) and the highly virulent strain (1137-C20) infecting a susceptible (Müller-Thurgau) and a tolerant (Regent) *V. vinifera* cultivar at 6, 12 and 24 hours post inoculation. Presented are means of three independent experiments. Error bars represent the SEM.

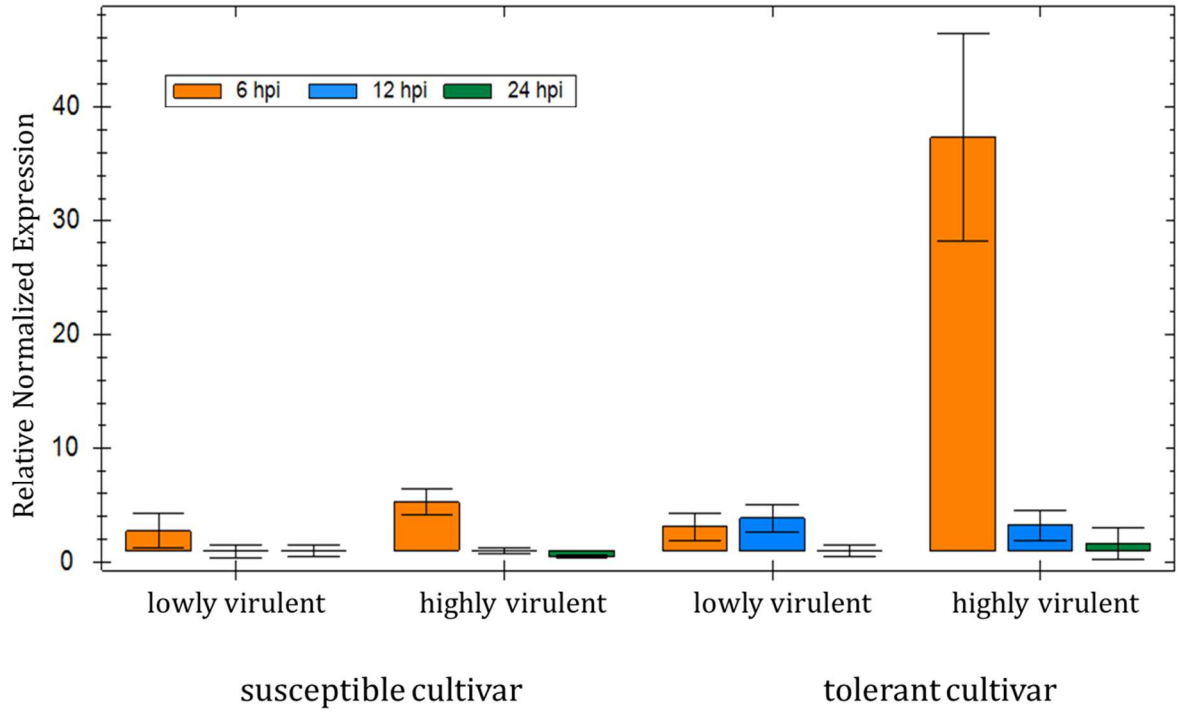

Figure 4.14: Relative normalized expression of the putative effector gene ***NLP 1*** on the lowly virulent strain (1135-F2) and the highly virulent strain (1137-C20) infecting a susceptible (Müller-Thurgau) and a tolerant (Regent) *V. vinifera* cultivar at 6, 12 and 24 hours post inoculation. Presented are means of two independent experiments. Error bars represent the SEM.

Elicitin like 2 showed a different picture (Fig. 4.15). In this case, the highest expression was found at 6 hpi like in the other effectors. In the other studied time points the expression was very low and it experienced no change through the infection process. An interesting fact here was that the highest expression was found in the highly virulent strain infecting the susceptible cultivar, thus contrasting the results with previous effectors. Another issue was that the expression of the lowly virulent strain remained almost the same, independently on which cultivar the infection took place. The highly virulent strain appeared not to have a host dependent regulation for this gene and it was simply highly expressed as in the other strain.

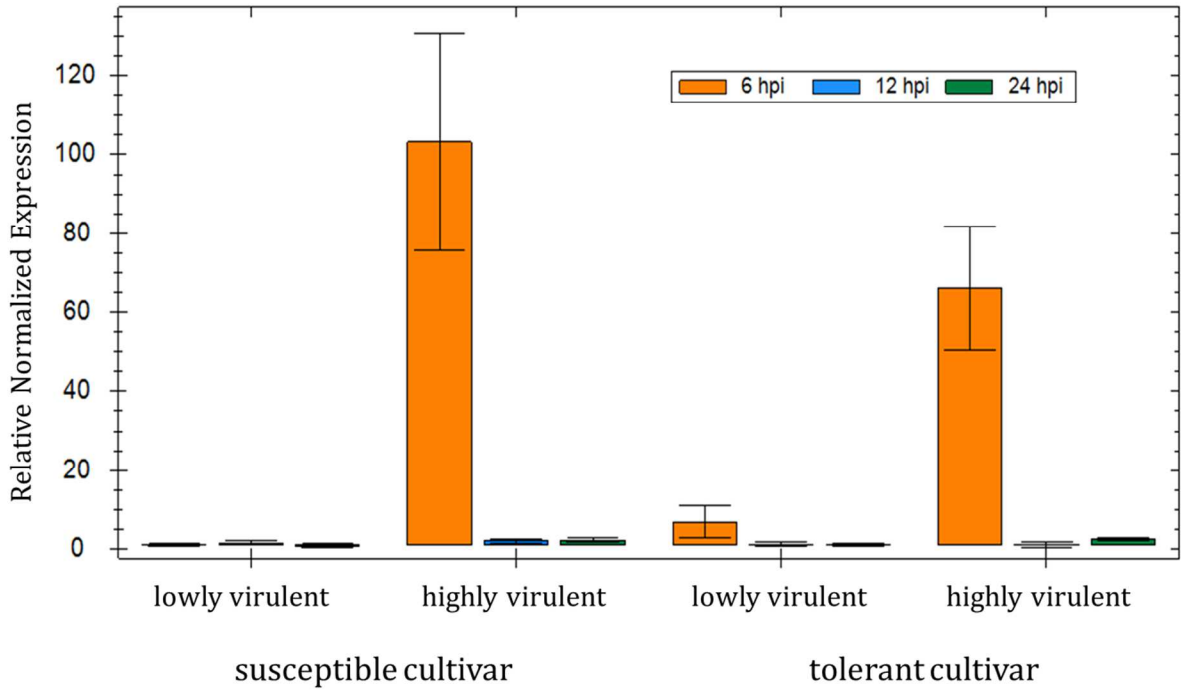

Figure 4.15: Relative normalized expression of the putative effector gene ***Elicitin like 2*** on the lowly virulent strain (1135-F2) and the highly virulent strain (1137-C20) infecting a susceptible (Müller-Thurgau) and a tolerant (Regent) *V. vinifera* cultivar at 6, 12 and 24 hours post inoculation. Presented are means of three independent experiments. Error bars represent the SEM.

In the case of *Glucanase inhibitor 2* the expression remained very low in the three studied time points in the susceptible cultivar. Nevertheless, on the tolerant cultivar both strains presented a high upregulation of this gene at 6 hpi, with no appreciable difference between them. At 12 hpi there was an important decrease on the expression level and at 24 hpi there was no more upregulation (Fig. 4.16).

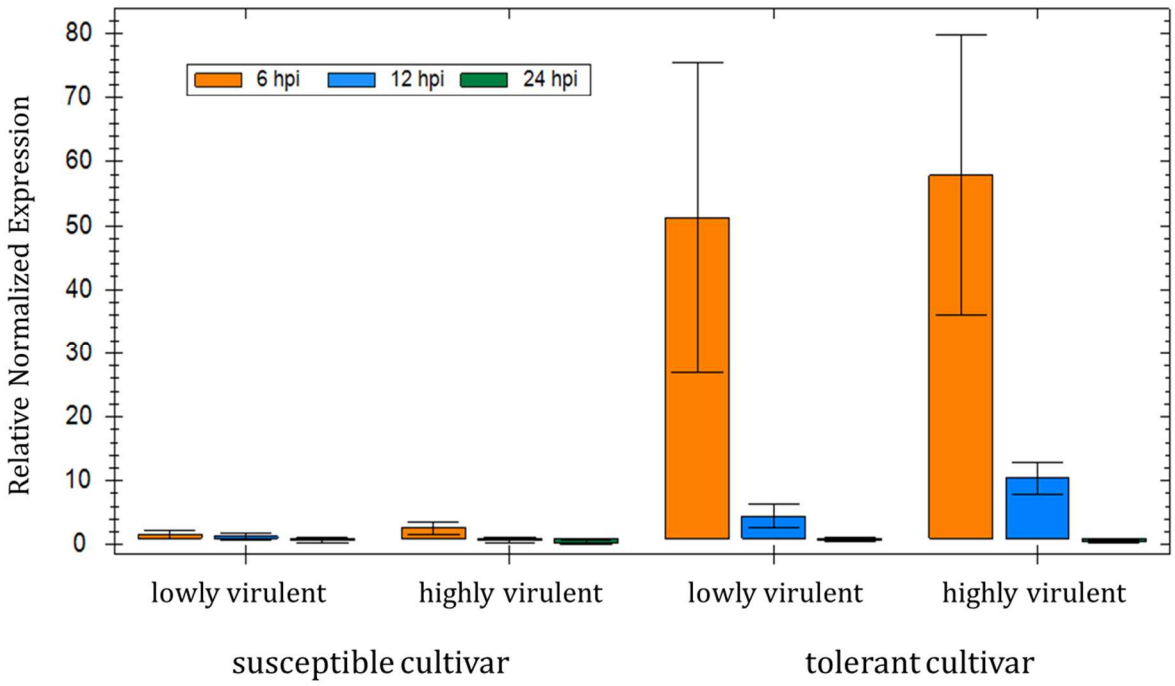

Figure 4.16: Relative normalized expression of the putative effector gene ***Glucanase inhibitor 2*** on the lowly virulent strain (1135-F2) and the highly virulent strain (1137-C20) infecting a susceptible (Müller-Thurgau) and a tolerant (Regent) *V. vinifera* cultivar at 6, 12 and 24 hours post inoculation. Presented are means of three independent experiments. Error bars represent the SEM.

The other gene of this group, *Glucanase inhibitor 4*, presented a different expression pattern (Fig. 4.17). As well as in the other genes, the expression peak was again found at 6 hpi and in this case not a big difference was found between all the host-pathogen combinations. It seems to be important for the infection process, especially at the beginning, but there was no host dependent regulation. The 1,*3 ß Glucanase 2* behaved differently. The highly virulent strain presented here the highest expression level, in particular at 6 hpi. It responded stronger to the tolerant cultivar than to the susceptible one, while the lowly virulent strain behaved similar in both cultivars (Fig. 4.18).

Contrary to the results from the previous section, none of the host-pathogen combinations showed a peak at 24 hpi. In all the selected effectors for which an upregulation occurred, the peak was found at 6 hpi and strongly decreased at 12 hpi.

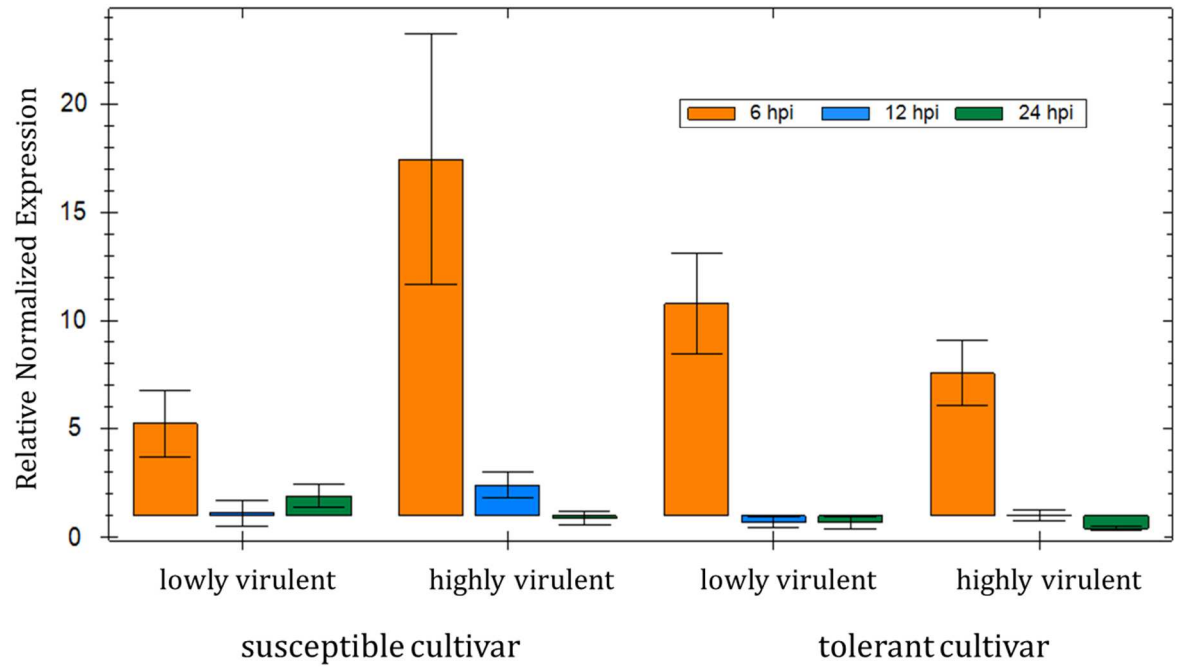

Figure 4.17: Relative normalized expression of the putative effector gene ***Glucanase inhibitor 4*** on the lowly virulent strain (1135-F2) and the highly virulent strain (1137-C20) infecting a susceptible (Müller-Thurgau) and a tolerant (Regent) *V. vinifera* cultivar at 6, 12 and 24 hours post inoculation. Presented are means of two independent experiments. Error bars represent the SEM.

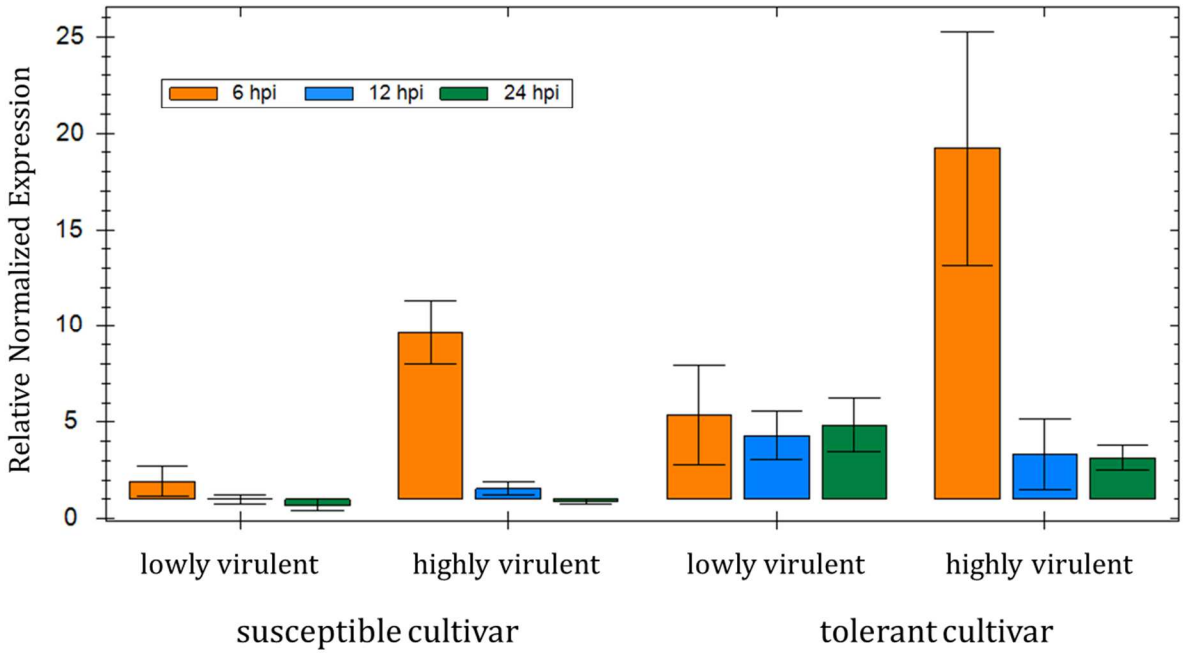

Figure 4.18: Relative normalized expression of the putative effector gene ***1,3 ß Glucanase 2*** on the lowly virulent strain (1135-F2) and the highly virulent strain (1137-C20) infecting a susceptible (Müller-Thurgau) and a tolerant (Regent) *V. vinifera* cultivar at 6, 12 and 24 hours post inoculation. Presented are means of three independent experiments. Error bars represent the SEM.

4.4 Discussion

4.4.1 A host-free system

Genetic studies in obligate biotrophic pathogens face many challenges. The dependence on a living host for the proliferation and development makes the maintenance and the handling of the pathogen a difficult task. To study metabolic characteristics, gene regulation, function of peptides and many other topics, a host-free system is desirable. Mestre *et al*., (2012) presented a methodology to study *P. viticola* gene expression in germinated zoospores. To induce the zoospore germination *in vitro*, NaCl 10 mM was applied. Our previous results (Fröhler 2012) suggested a higher effectiveness of $CaCl_2$ to achieve zoospore encystment and germination. In the subsequent work of Bendel (2013) and in the current study, for at least three different single sporangium strains of *P. viticola* as well as for field isolates of three other oomycetes, it was shown the advantage of $CaCl_2$ over NaCl in terms of time and efficiency to induce encystment and germination of zoospores. This methodology constitutes a promising tool to study the early developmental stages of the pathogen before establishing direct contact with the host. An advantage of this system is the ability to study differences between strains in the laboratory without depending on living material of the host. This becomes particularly important for biotrophic oomycetes limited to perennial hosts which cannot be easily cultivated during winter in growing chambers.

4.4.2 Effectors at the genetic level

Our efforts to find out differences between the five selected strains of the pathogen spurred us to dig deeper into the genetic level. Interestingly, all the 25 effector genes screened were present in the genome of the five strains which was against our initial assumption. It is known that the pool of effectors of an isolate, including the specific alleles, can enable a strain to overcome the resistance of specific cultivars. The latest study on the secretome of *P. viticola* showed that not all studied isolates presented the same repertoire of effectors and that different alleles existed between them (Yin *et al*., 2015). A high pool of effectors in the genome of an isolate might be responsible for a higher fitness. Possessing effectors of functional redundancy may allow a pathogen to lose or inactivate an effector in order to avoid detection by host resistance (R) proteins without compromising its virulence (Birch *et al*., 2008, Raffaele & Kamoun 2012). It is

also believed, that more competitive *P. viticola* genotypes are those possessing a more complex arsenal of effectors what makes them able to stop the plant defense mechanisms (Roatti *et al*., 2013). Future studies could reveal if differences in the sequence of these genes are physiologically important.

4.4.3 Gene expression in germinated spores and infected leaves

Since the knowledge of these effectors was limited, and just partial sequences were known, functional studies were not possible. The next step in the differentiation of the strains was the study of the gene expression. Using the early stadium of the germinated spores, it was possible to compare the expression of effector genes between the strains. From the three studied effector genes at this point, just *RXLR 1* showed a high level of expression. Surprisingly, there was again no difference between the five strains. A remarkable issue was the extremely high expression of this gene on the germinated spores, suggesting an important role of this gene, especially in the early events. The gene expression on infected leaves at 96 hpi was much lower than in the germinated spores, which made us focus more on the earlier stages. On the susceptible cultivar Müller-Thurgau, the five strains exhibited the same behavior and the gene expression was similar as well. This issue supports the assumption that the expression of effector genes might be affected by a host response and that the pathogen can regulate it establishing a fine communication system. The analysis of the gene expression on a tolerant strain Regent confirmed this idea.

4.4.4 The host-pathogen combination

The particular host-pathogen combination found between two strains with different virulence (1135-F2 and 1137-C20) and a tolerant host (Regent) enabled us to go further in the analysis of effector gene expression. In the way to find a relationship between the expression of effector genes and its effect in the virulence of a specific strain, this combination was found especially interesting. The first six hours after inoculation seemed to be a very critical time lapse, in which a very high gene expression was found. Effectors might play a decisive role at the first encounter between host and pathogen to enable a compatible infection. Probably, the battle between the plant and the pathogen is decided in the first hours of contact, and the later events may just be an answer to what occurred earlier. Previous studies of the host response to downy mildew infection

are in accordance with this theory. It has been shown that a rapid recognition of the pathogen, together with an induction of the plant defense mechanisms in the early stages of infection, are key points for resistance (Gindro *et al*., 2003, Kortekamp & Ziprian 2003, Díez-Navajas *et al*., 2008). Additional evidence of how decisive are the early events for the infection was found on a gene expression study in *V. riparia* where the resistance response against *P. viticola* began within the first 24 hpi (Polesani *et al*., 2010). Nevertheless, it has also been found that gene expression shows different patterns as they colonize the plant (Schornack *et al*., 2009) and different effectors are upregulated at different points of the colonization process (Yin *et al*., 2015).

4.4.5 Tissue colonization and gene expression

The biggest differences on gene expression between the high and the low-virulent strain were found in the tolerant cultivar at 6 hpi; however, microscopy did not show any difference until 48 hpi between both strains on Regent. This is a hint that early regulation of gene expression might be anatomically reflected many hours later. Other authors have also reported that in the early stages of infection, *P. viticola* develops similarly on susceptible and resistant *Vitis* species (Unger *et al*., 2007, Díez-Navajas *et al*., 2008, Liu *et al*., 2015). The observed reduction in hyphal development in Regent (Fig. 4.11) was reported as a defense mechanism in the resistant cultivar Solaris (Boso & Kassemeyer 2008) and in resistant Asiatic *Vitis* species (Yu *et al*., 2012). As the abundance of hyphae in the mesophyll might be a triggering signal for sporulation (Unger *et al*., 2007), the growth reduction observed in Regent could be an efficient mechanism to avoid the infection spread without going through plant cell death.

4.4.6 Effector gene expression

Analyzing the gene expression of both strains, an important difference was revealed (Fig. 4.12). While the highly virulent strain strongly upregulated *RXLR 1* at 6 hpi, the same process did not occur in the lowly virulent strain until 24 hpi. Intriguingly, a similar expression was not found in the second experiment 24 hpi (using the 0 hpi as an 'untreated sample' for normalization) for any of the studied effectors. It is clear that in such a complex pathogen, small differences between experiments might drastically affect the results. Therefore, a very standardized protocol is required to assure

repeatability, although in a biotrophic system, sources of variation will be always present.

The faster reaction of the highly virulent strain might be the key to a successful infection. Results from the plant side correlate with this theory. Polesani *et al*., (2010) reported that *V. riparia* answered sharper to the infection compared to a susceptible cultivar of *V. vinifera* in terms of upregulation of disease related genes. Studying the resistant cultivar Bianca (*Rpv3*), it was found that early responses of the host were critical to stop the invasion, mainly in those cells directly under the stomata, which are the first in establishing contact with the pathogen through haustoria (Casagrande *et al*., 2011). An early up-regulation of the synthesis of jasmonic acid has also been reported in a downy mildew resistant *Vitis* genotype (Figuereido *et al*., 2015). To face such an early defense response of the plant, successful isolates of *P. viticola* might be able to respond as well in a very rapid way.

The screening for further virulence-related effectors generated interesting results in our second experiment. *RXLR 1* remained as an outstanding gene due to the very high expression shown, especially 6 hpi in the high-virulent strain when infecting Regent. The case of *NLP 1* was similar, despite the induction fold was lower. *1,3 ß Glucanase 2* showed also a similar pattern. The high expression of these effector genes in the virulent strain on Regent supports the assumption of their role in the pathogenic process. *Glucanase inhibitor 2* and *4* showed important up regulations at 6 hpi, especially in Regent. Glucanase inhibitors are part of a well-known mechanism used by pathogens to defend themselves against pathogenesis-related (PR) proteins from the plants (Kamoun 2006). A potential contribution in the virulence of this effector under these conditions should be further studied. *Elicitin like 2* showed a very high upregulation but just on the highly virulent strain at 6 hpi. This activity supports the assumptions of the crucial role on early events.

4.4.7 The plant defense reaction

The expression patterns of the *stilbene synthase* and *metacaspase 2* and *5* analyzed in the cooperation framework in the KIT in Karlsruhe did not show any conclusive pattern in the early stages analyzed and should be subjected to further analysis. It was detected a higher expression of *NBS-LRR2* in the tolerant cultivar compared with the susceptible

one (personal communication Peijie Gong). A protective activity of proteins from this family has been confirmed after downy mildew infection in grapevine (Fan *et al*., 2015). Does the highly virulent strain possess slightly mutated effectors allowing it to infect while triggering a lower defense reaction? Are the effectors of the highly virulent strain able to modify proteins in a way that they are not detected by the surveillance system of the plant, or at least not fast enough? Or does the virulent strain bear other effectors able to down-regulate the plant defense? Further studies should address these questions to clarify the mechanisms used by *P. viticola* to successfully infect resistant *Vitis* hosts.

4.4.8 Where are we now?

At the beginning of this work, a very limited number of putative effector genes were known, for which just a partial nucleotide sequence had been discovered (Mestre *et al*., 2012). Nevertheless, a very recent paper presented a list of 51 RXLR effectors of *P. viticola* including their complete nucleotide sequences (Yin *et al*., 2015). Comparative analysis could reveal if differences in the sequence are related to a higher or lower virulence. Higher efforts in the sequencing of strains with different virulence are still necessary to achieve these goals.

The host-free system studied showed to be a useful tool to assess the early and susceptible stages of the pathogen life cycle. Advances in the research to identify the function of effector genes would enable a screening to find interesting strains which upregulate decisive genes. The leaf disc test is another methodology that permits the study of gene expression at later time points after the inoculation. Different effector genes suffer upregulation at different point of the leaf colonization. It is still necessary to study in detail the course of the infection to determine if there are key points where the pathogen inactivates the plant defense reaction, or if there is a constant down regulation process.

The advantage of a host-pathogen combination to study gene expression and the involvement of effectors on the virulence of strains has been recently proven by other authors (Li *et al*., 2015). This assay contributes to gain knowledge to which extent effectors are highly expressed in a compatible infection of a *P. viticola* strain infecting a tolerant grapevine genotype. Screening for new interesting host-pathogen combinations is been carried out and future studies would rely on such interactions to search for

explanations of compatible and incompatible interactions. How do effectors exert their activity in the plant? Which effectors play a decisive role in establishing a compatible interaction? How early begins the interaction between plant and pathogen? This and many other questions remain unanswered and require future investigation to make improvement in the control of this important pathogen.

Epilogue

At the beginning of this work, no suitable test system to classify isolates of *P. viticola* had been reported, although it has been known for a long time that populations with different virulence and different tolerance to fungicides exist in the field. Therefore, the development of such a methodology to characterize strains of *P. viticola* according to their virulence on standardized host genotypes was a very important contribution. As lined out in chapter 1 and published in Gómez-Zeledón *et al*., (2013), the diversity in field populations often requires the selection of single sporangium strains in order to assess the full spectrum of pathogen genotypes present in a vineyard. Using a leaf disc bioassay was the least laborious and fastest method to classify pathogens by evaluating two parameters, sporulation intensity and host necrotic reaction. This methodology permitted, as well, a characterization of the fungicide resistance of the studied strains. Again it was shown that testing bulk samples may sometimes be misleading. As shown in Fig. 1.3, some fungicide resistant genotypes of low representation in a population may be overseen when testing bulk samples, whereas selection of genetically homogenous strains revealed their presence. This system therefore provides an important tool for researchers, breeders and farmers and has already been used by other groups to characterize isolates of the pathogen.

Further work will be necessary in the future to extend this bioassay by broadening the range of host genotypes in order to increase the resolution of the pathotype classification. As shown in chapter 2, *Vitis* species from North America as well as from Asia provide a rich source for breeders to seek for better resistance against downy mildew. Interestingly, not all species tested from North America, which a long-lasting co-evolutionary history with the pathogen had shown high resistance. On the other hand, some Asiatic species which where thought not to have developed resistance to a natural North American endemic pathogen, did not allow colonization. It will be an interesting task for further research to investigate such incompatible host-pathogen pairs and to unravel the mechanisms behind it.

The high genetic variability reported in chapter 3 was found using molecular markers in the tested strains. This confirmed the results from the bioassays, and emphasized, how relevant the achievement of homogeneous material through single sporing for this pathogen is. Even though for other fungal species the single sporing is a routine

procedure, in oomycetes, it is still not commonly practiced due to the difficulties to achieve infections from single spores or sporangia. The identification of highly susceptible host genotypes such as *V. betulifolia* in the infection assays will help to improve the rate of successful infections with single sporing technique. Many authors still rely on single sporangiophores strains, even if it is still not clear if all sporangia have the same genetic material. Further studies using molecular markers might help to clarify this topic.

A very interesting approach to study the expression of effector genes in strains with different virulence was constituted by the host-pathogen combination. With help of this methodology, promising expression patterns were found that might reveal the mechanism behind the ability of some strains to overcome the resistance from the plant in the future. The possibility to have homogeneous characterized strains, for which the reaction produced in different hosts was known, was an important milestone that allowed us to carry out this study. To achieve a better understanding of the mechanisms responsible for a specific reaction on a *Vitis* host, cooperation was established with the KIT in Karlsruhe, and the reaction of the plant was examined. In concordance with other studies, it was possible to identify at least one gene with an interesting expression pattern that might be involved in the resistance of the grapevine cultivar Regent. More genes from the side of the plant should be the subject of future studies to get a better picture of the reaction of the plant against the infection of *P. viticola*.

The role of some interesting effectors expressed by the pathogen following a pattern should be analyzed in more detail. Especially outstanding was the high expression of a gene bearing a *RXLR* motif in early stages, which might be related with a higher virulence in specific strains. Other authors have previously shown interest for this kind of studies, finding important results in this field analyzing this group of effectors. Approaches to discover new effectors have begun and should continue to gain a more complete knowledge of the secretome of the pathogen. The role of specific effectors in the plant would be the next step to understand exactly what happens when the oomycete infects the plant. Transient expression analyses have made this possible for some genes, but still much work needs to be done in this direction. The study of the early developmental stages of the pathogen using *in vitro* germinated spores has proven to be an advantageous approach. This work showed that even without contact with the plant, strains of the pathogen react differently, what might imply an advantage of

overexpressing certain genes at early stages. Future studies need to consider this tool to gain more knowledge in the first infection stages of the oomycete.

Fluorescence microscopy was important to follow the development of the pathogen inside the leaf, or even before as the spores begin to encyst and germinate. Many other authors have confirmed the importance of this kind of studies to reveal which processes are being developed in the plant, and how the pathogen behaves in different conditions or in different hosts.

After answering some of the questions posed at the beginning of this study, many new questions arouse, showing how complicated and challenging this pathosystem is. Some years after oomycetes were listed as the nightmare for geneticists, this and many other studies reflect what other authors have recently expressed: oomycetes constitute a very important tool to increase the knowledge on plant-pathogen interactions.

Summary

The downy mildew of grapevine, *Plasmopara viticola*, is one of the most important pathogens in viticulture. Its genetic diversity had been assessed in some previous studies using molecular markers, but the diversity of the infection behavior has not yet been addressed adequately. Therefore, the development of a fast, reliable and uncomplicated assay to screen for pathogen phenotypes on host with different resistance levels was a major task of this work. A leaf disc test was proposed, evaluating sporulation and necrosis produced by the pathogen on *Vitis* plants with different susceptibility. Using this bioassay, interesting strains were assessed and kept for future studies. The urgent need to work with genetic homogeneous inoculum was shown, because the assays revealed a high phenotypic diversity in isolates collected from the field as a bulk sample. Hence, a cloning technique to obtain single sporangium strains was found useful to avoid working with mixed genotypes.

The leaf disc bioassay also allowed screening for fungicide resistance in *P. viticola* populations. Isolates resistant to dimethomorph and metalaxyl, two important fungicides for oomycetes control, were detected. Higher resistance was associated with fields were the fungicide application was high as well. Some strains were even resistant to doses where the fungicide exhibits phytotoxic activity to grapevine. The approach of characterizing *P. viticola* pathotypes on different host plants of *Vitis vinifera* cultivars and *Vitis* species from North America and Asia revealed a broad spectrum of fully susceptible to completely resistant reactions. This information is of direct practical value in future plant breeding programs, but also provides the chance to select specific host-pathogen combinations to study the mechanisms of resistance or susceptibility. Fluorescence microscopy revealed how the infection progress of highly and lowly virulent strains advance in tolerant and susceptible hosts, and which points of the infection are interesting for future studies. On the molecular level, effectors were investigated to trace their possible involvement in the infection process. It was found that *RXLR 1*, *NLP 1*, *Elicitin like 2*, *Glucanase inhibitor 2* and *4* , and *1,3-ß Glucanase 2* are candidates which are upregulated in the earliest infection stages. Following the here established methodology and suggested strategy it should be possible in the future to get a better insight in the mechanisms of infection and resistance of grapevine downy mildew.

Zusammenfassung

Der Falsche Mehltauerreger von Wein, *Plasmopara viticola*, ist eines der wichtigsten Pathogene im Weinbau. In vorherigen Studien wurde die genetische Diversität diese Pathogens mittels molekularer Marker evaluiert, jedoch erfolgten keine aussagekräftigen Untersuchungen bezüglich der phänotypischen Diversität. Aus diesem Grund war die Entwicklung einer unkomplizierten Methode, verschiedene Genotypen des Pathogens durch Biotests zu unterscheiden, eines der Hauptziele dieser Arbeit. Hierzu wurde ein Blattscheibentest mit unterschiedlich anfälligen *Vitis* Kultivaren entwickelt. Für die Bewertung der Pathogene wurden Parameter wie die Art der verursachten Sporulation sowie Nekrosenbildung ermittelt. Durch Verwendung dieses Biotests, konnten interessante Pathogenstämme identifiziert und für zukünftige Studien gezielt vermehrt werden. Hierbei zeigte sich die dringende Notwendigkeit, mit einem genetisch homogenen Inokulum zu arbeiten, da eine hohe phänotypische Diversität in Feld Isolaten experimentell nachgewiesen wurde. Um die Arbeit mit Mischisolaten zu vermeiden wurden durch ein Klonierungsverfahren Stämme aus einzelnen Sporangien herangezogen.

Der entwickelte Blattscheibentest ermöglichte auch das Screening auf Fungizid Resistenzen in *P. viticola* Populationen. Resistente Stämme gegen Dimethomorph und Metalaxyl, zwei bedeutende Fungizide für die Bekämpfung dieses Oomyceten, wurden hierbei nachgewiesen. Dabei wurden hohe Resistenzen mit Feldern, in denen die Fungizid Anwendung ebenfalls hoch war, assoziiert. Es wurden sogar einige Stämme identifiziert, die gegen sehr hohe Dosen, zum Teil bereits im phytotoxischen Bereich für die Weinrebe, Resistenz aufwiesen. Die *P. viticola* Pathotyp Charakterisierung mit verschiedenen *Vitis vinifera* Kultivaren sowie *Vitis* Arten aus Nord Amerika und Asien zeigte ein breiteres Spektrum von komplett anfälligen bis zu komplett resistenten Reaktionen. Diese Information kann in künftigen Züchtungsprogrammen direkt angewendet werden. Zusätzlich ermöglicht diese Methode auch die Auswahl interessanter Wirt-Pathogen Kombinationen. Diese Kombinationen sollen genutzt werden, um die Mechanismen, welche für die Resistenz verantwortlich sind, zu untersuchen. Fluoreszenzmikroskopische Untersuchungen zeigten wie sich die Infektionsverläufe von hoch und niedrig virulenten Pathogenstämmen in toleranten und anfälligen Wirten verbreiten. Hierbei konnten auch die, für weitere Studien

interessanten Zeitpunkte der Infektion, ermittelt werden. Einige der, so ermittelten, Zeitpunkte, sowie sehr frühe Stadien der Infektion, wurden auf molekularer Ebene untersucht. Anschließend wurden verschiedene Effektoren betrachtet um deren Beteiligung während des Infektionsprozesses nachzuvollziehen. Es wurde festgestellt, dass *RXLR 1*, *NLP 1*, *Elicitin like 2*, *Glucanase inhibitor 2* and *4*, and *1,3-ß Glucanase 2* Kandidaten für Gene sind, die in frühen Entwicklungsphasen der Sporen hochreguliert werden. Die Benutzung der hier etablierten Methode und vorgeschlagene Strategie solle künftig ein besseren Einblick des Infektionsmechanismus und Resistenz gegen Falschen Mehltaus ermöglichen.

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List of abbreviations

bp	base pair
BLAST	Basic Local Alignment Search Tool
CAA	carboxylic acid amides
cDNA	complementary DNA
cv	cultivar
DNase	deoxyribonuclease
DEPC	Diethylpyrocarbonate
DIM	dimethomorph
EST	expressed sequence tag
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
hpi	hours post inoculation
HR	hypersensitive reaction
inc	incorporated
MET	metalaxyl-M
MultiNa	Microchip electrophoresis system for DNA/RNA analysis
NCBI	National Center for Biotechnological Information
PTI	PAMP triggered immunity
PAMP	pathogen associated molecular pattern
PR	pathogenesis-related
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase PCR
SEM	standard error of the mean
SNP	single nucleotide polymorphism
SSR	simple sequence repeat
AES	*Vitis aestivalis*
AM	*Vitis amurensis*
BET	*Vitis betulifolia*
CIN	*Vitis cinerea*
COI	*Vitis coignetiae*
DAV	*Vitis davidii*
RIP	*Vitis riparia*
RUP	*Vitis rupestris*
CAB	*Vitis vinifera* cv. Cabernet Cortis
MT	*Vitis vinifera* cv. Müller-Thurgau
REG	*Vitis vinifera* cv. Regent
SYL	*Vitis vinifera* ssp. *Sylvestris*
WBI	Weinbauinstitut Freiburg

Appendix

Table A.1: Accession number of the analyzed effectors and reference genes.

Analized fragment	Accession number (NCBI)
Glucanase inhibitor_1	HE582132
Glucanase inhibitor_2	HE582107
Glucanase inhibitor_3	HE582041
Glucanase inhibitor_4	PVS1008
RXLR_1	HE582030
RXLR_2	PVS0817
Elicitin like_1	HE582038
Elicitin like_2	HE582165
Endo-1,3 ß Glucanase_1	HE582100
Endo-1,3 ß Glucanase_2	HE582125
Endo-1,3 ß Glucanase_3	PVS0495
Endo-1,4 ß Glucanase_1	HE582050
Secretory protein OPEL_1	PVS0463
Secretory protein OPEL_2	PVS0049
Pectinesterase_1	HE582127
Pectinesterase_2	PVS0290
Pectinesterase_3	PVS0401
Transglutaminase elicitor_1	HE582159
Transglutaminase elicitor_2	HE582090
Acidic chitinases	HE582051
Catepsin-like cystein protease_1	HE582131
Catepsin-like cystein protease_2	PVS0379
Cystatin like protease inhibitor_1	PVS0212
Cystatin like protease inhibitor_2	PVS0283
Kazal like protease inhibitor	HE582205
Plasmopara viticola Actin	HE582092
Plasmopara viticola Tubulin	HE582072
Plasmopara viticola Elongation Factor 1 α	EF426554.1

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Curriculum vitae

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Academical career

2001-2007	Bachelor in Biology, University of Costa Rica
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2003-2004	Botany for biologists I and II (2 years, teaching)
2004	Micro-techniques, microscopical preparations (6 months, teaching)
2005	Molecular biology, virus detection (6 months, research)
2006	Electronic microscopy, with emphasis in SEM (6 months, research)
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2007-2010	Plant biotechnology, *in vitro* culture, secondary metabolite production, plants micro propagation (3 years, M.Sc. research)
2008	Plant physiology (6 months, teaching)
2008	Anatomy and microscopy of oil palm embryos (6 months, research)

2009 *In vitro* tissue culture (6 months, teaching)

2009-2010 Molecular pathogens detection (1 year, research)

2010-2011 Micro propagation of medicinal plants (6 months, research)

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2015 Microscopy course (light and electronic) (4 months, teaching)

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Scientific publications

Publications

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Participation in workshops and congresses

Oral presentations

Gómez-Zeledón, J., Jiménez, V., 2011. I*n vitro* induction of *callus* in *Hylocereus costaricensis* (Cactaceae). 8th Congress of Plant Biotechnology, BIOVEG. Bioplants Center, Ciégo de Ávila, Cuba, May 2011.

Gómez-Zeledón, J., Jiménez, V., 2011. *In vitro* production of pigments in *callus* of pitahaya var. Orejona (*Hylocereus* sp.) using different elicitors of biotical and abiotical origin and analysis of the pigments produced. 8th Congress of Plant Biotechnology, BIOVEG. Bioplants Center, Ciégo de Ávila, Cuba, May 2011.

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Gómez-Zeledón, J., Becker, S., Spring, O., 2015. Putativ effectors in strains of downy mildew with different virulence. 10th Rhein-Wein Symposium. Institute for Technology. Karlsruhe, Germany, May 2015.

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Poster presentations

Gómez-Zeledón, J., Murillo, A., 2011. *Fusarium proliferatum* detection in rice on Costa Rican fields. 8th International Congress of Plant Biotechnology: BIOVEG. Bioplants Center. Ciégo de Ávila, Cuba, May 2011.

Gómez-Zeledón, Romero, R., 2011. Development of a Biotechnological package for the production of dry plant and extracts of *Justicia pectoralis* and *Lippia alba* with high concentration of active principles for its use in the pharmaceutical and nutraceutical national industry. 8th International Congress of Plant Biotechnology: BIOVEG. Bioplants Center. Ciégo de Ávila, Cuba, May 2011.

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Gómez-Zeledón, J., Kaiser, M., Spring, O., 2015. Effector gene expression in *Plasmopara viticola* strains. 36th New Phytologist Symposium. Cell biology at the plant-microbe interface. Munich, Germany, 29 November–1 December 2015.

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Melanie Fröhler, B.Sc. (2012) Einfluss physikalischer und chemischer Faktoren auf frühe Infektionsstadien von *Plasmopara viticola*, dem falschen Mehltau des Weines. University of Hohenheim.

Nele Bendel, B.Sc. (2013) Optimierung der Versuchsbedingungen zur Erzeugung von Einzelsporeninfektionen bei *Plasmopara viticola*. University of Hohenheim.

Sandra Becker, M.Sc. (2015) Putative Effektoren von *Plasmopara viticola* in Sporangienklonen unterschiedlicher Pathogenität. University of Hohenheim.

Markus Kaiser, B.Sc. (2015) Virulenztypisierung von *Plasmopara viticola* Isolaten auf Rebarten unterschiedlicher Resistenz. University of Hohenheim.