An Extended Leaf Disc Test for Virulence Assessment in *Plasmopara viticola* and Detection of Downy Mildew Resistance in *Vitis*

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Abstract

Viticulture is continuously suffering significant economic loss from downy mildew epidemics. Although the infection biology of the pathogen *Plasmopara viticola* is well understood, little is known regarding the population diversity of this oomycete and on the mechanisms responsible for compatible or incompatible reactions with different host genotypes. The discrimination of strains with different virulence is a fundamental step for the assessment of pathotypes in local populations and could help to develop measures for preventing economic loss. We here describe an extended and slightly modified bioassay for the assessment of virulence by means of sporulation intensity. Simultaneously, the necrotic reaction of host genotypes is considered and allows identification of different resistance strategies. Comparison of infections symptoms of 12 host genotypes after inoculation with five different single sporangium strains of *Plasmopara viticola* showed that: 1. resistance diversity is high in some *Vitis vinifera*. Cultivars; 2. *Vitis riparia* showed the strongest resistance amongst the four tested North American species; 3. Asian *Vitaceae* harbor fully resistant as well as highly susceptible genotypes. In addition, microscopic investigation of inoculated leaf discs from *Vitis amurensis* and *Ampelopsis japonica* unraveled, that despite lack of sporulation, a mycelium may grow to a certain point in resistant hosts. The necrotic reaction in *V. amurensis* indicates a different mechanism of resistance when compared to *A. japonica*, where no necrosis was found.

Keywords: Host-pathogen interaction; Pathogen phenotyping; Grapevine downy mildew; *Vitis*; Leaf disc bioassay

Introduction

The downy mildew of grapevine is one of the most destructive diseases in viticulture resulting in severe epidemics and enormous economic costs. The causal agent is *P. viticola* (Berk. and Curt.) Berl. and de Toni, an obligate biotrophic oomycete of the Peronosporaceae family, which was introduced to Europe from North America in the 1870s [1]. Due to the lack of natural resistance in the European grapevine *Vitis vinifera* against the new pathogen, chemical measures for disease control soon became necessary. Currently, increasing amounts of fungicides and multiple applications throughout the season are necessary for adequate disease control [2] and consequently, fungicide resistance is frequently found in pathogen populations of commercial vineyards [3]. On the other hand, sources for natural resistance are present in North American and Asiatic wild *Vitis* species such as *V. riparia*, *V. ruprestris* or *V. amurensis*, and have been used for breeding since the 19th century [1,4,5]. However, considering the economic impact of *P. viticola*, there is still very limited knowledge on mechanisms relevant for the pathogenesis of this oomycete, and the development of alternative control methods for an integrated pest management in grapevine downy mildew would be highly desirable [6].

To gain a better understanding of the infection process, it is essential to understand both sides: the attacking strategy of the biotrophic pathogen as well as the defense reaction of the host plant. In contrast to some other commercially relevant biotrophic oomycetes such as *P. halstedii* on sunflower or *Bremia lactucae* on lettuce, a system for virulence assessment and classification of pathotypes for *P. viticola* was missing. This changed recently with the publication of a standardized leaf disc bioassay on defined hosts for the characterization of the virulence of grapevine downy mildew isolates [7]. Screening of field populations showed inconstant reactions, thus indicating genetic inhomogeneity within the sporangia sample of a single field. Subsequent testing of single sporangium strains from such field isolates produced varying infection reactions on selected hosts, thus confirming the presence of multiple pathotypes in local populations and the high genotypic diversity reported from genotypic analysis [8-10]. This underlines the importance of working with genetically homogeneous strains of the pathogen when investigating virulence behaviour or resistance reactions. In such a way, a leaf disc virulence test was recently employed to screen *P. viticola* strains infective to *V. amurensis* and subsequently used selected strains to search for downy mildew resistance genes [11].

The discrimination of strains with varying degrees of virulence is a fundamental step for the phenotypic characterization of *P. viticola*. This can help to monitor the occurrence of new or particularly virulent phenotypes of the pathogen, thus improving the possibilities for applying control measures. Additionally, the leaf disc bioassay for virulence assessment provides the possibility to screen for specific host-pathogen combinations which may help to identify new sources of resistance for breeding new cultivars. However, when using our assay on a broader range of pathogen isolates, it became clear that sporulation and necrosis should be treated as independent features in the evaluation system. Therefore, in the present study, a modified classification is proposed which improves the possibility for genotype discrimination when considering the reaction of the plant (necrosis) and the reaction of the pathogen (sporulation) separately and applying different symbols for infection phenotypes. In addition to changes in scoring symptoms, an extension of the host differentials is suggested. Compared to our previous assay [3], we increased the number of *V. vinifera* genotypes to four and added six more North American and...
Asiaic wild species in order to broaden the basis of genetically differing resistance mechanisms.

Material and Methods

The characterization system

For the phenotypic characterization of *P. viticola* strains, the leaf disc inoculation test published in 2013 [3] was applied with some modifications: i) to inoculate the leaf discs, 1000 sporangia were used instead of 10000; this reduced the amount of sporangia required for the assay without diminishing the infection efficiency; ii) the evaluation of the infection was performed 10 days after inoculation instead of 14 days with the effect that the risk of undesired secondary infections by other microorganisms was reduced. The characterization system was refined, separating the assessment of the sporulation and the necrosis using different symbols (Table 1). To evaluate the level of necrosis produced, the following symbol code was used: (+++) strong, (+++) moderate, (+) weak and ( ) absent (Figure 1).

North American and Asiatic species

Five single sporangium strains of *P. viticola* (Berk. and Curt.) Berl. and de Toni cloned from field isolates of different wine-growing regions were selected for this study. Origin/host cultivar of the strains correspond as follow: 1117-A21: Colmar, France/Cabernet Sauvignon, 1135-F2: Freiburg, Germany/Müller-Thurgau, 1136-A15: Pfaffenweiler, Germany/Regent, 1137-C20: Pfaffenweiler, Germany/Gutedel, 1191-B11: Laufen, Germany/Lemberger. These strains were selected based on their characteristic reaction on six different grapevine genotypes of three regions were selected for this study. Origin/host cultivar of the Berl. and de Toni cloned from field isolates of different wine-growing regions were considered: *Vitis riparia* sylvestris. In the second group, *V. vinifera* (ID: 6548), *Vitis rupestris* (ID: 5888), *Vitis cinerea* (ID: 6128) and *Vitis aestivalis* (ID: 5911). In the third group, four Asiatic species were included: *Vitis coignetiae* (ID: 6542), *Vitis amurensis* (ID: 6540), *Vitis betulifolia* (ID: 6126) and *Ampelopsis japonica* (ID: 6544). The reaction of *V. jacquemontii* (ID: 5883) was analysed as well, but for practical reasons this species was not included in the routine test system. Plants grown outdoors in the botanical garden were selected to harvest leaves for the test. Leaves between the fourth and the seventh from the shoot tip were used for the bioassay. Experiments were repeated at least twice.

Microscopy

Inoculated leaves of *V. vinifera* cv. Müller-Thurgau, *V. amurensis*, *A. japonica* and *V. jacquemontii* were analyzed microscopically to study the reaction at the tissue level. Leaf discs were fixed in 70% ethanol for 24 hours and clarified using 5% KOH at 95°C for five to seven hours. After washing the discs, aniline blue (0.05%, 0.0067M, K,HPO₄, pH 9-9.5) was added 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 under an Axioplan microscope (Zeiss, Oberkochen). A fluorescence filter (Zeiss, filter II, 02 /G365, excitation: 365 nm) was employed under UV light to observe the discs. Pictures were taken using a digital camera (Canon Power Shot A640). The software LAS 4.6.1 (Leica Microsystems, Switzerland) was used for overlaying pictures.

Results

The characterization system

The leaf disc assays with an extended range of *Vitis* genotypes showed that in some cases (e.g. *V. riparia*, or *V. aestivalis*) the evaluation, when based predominantly on the degree of sporulation, would classify some phenotypes in the same category, although the necrotic reaction of the host clearly indicated differences in the host-pathogen interaction (Figure 2). This problem was overcome by independently evaluating sporulation and necrosis (Figure 1). Using the five previously categories established for pathogen aggressiveness ranging from unlimited (A) to no sporulation (E), the resistance reaction of the plant was categorized with four additional classes that ranked necrosis from strong (+++) to absent ( ) (Table 1). This alteration not only refines the classification of phenotypes, but also provides better information for the selection of host genotypes according to specific resistance reactions.

In the case of *V. riparia*, for instance, it becomes clear that despite the lack of sporulation in all five tested strains, three strains caused necrosis in the tissue, while the two others caused no visible reaction in the host (Figure 2). Similarly, all tested strains would be classified as type E (no sporulation) on *V. amurensis* and *A. japonica*, while strong sporulation and necrosis in two separate categories.

### Table 1: Description of the modified system for the phenotypic characterization of *Plasmopara viticola* isolates according to the infection reaction produced on *Vitis* leaf discs 10 days after inoculation. Sporulation is categorized using colors/letters and necrosis using the symbol (+).

| Code | Category | Sporulation 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 | Complete absence of necrotic reaction |

Figure 1: Diagram showing the characterization system according to the intensity of sporulation and necrosis in two separate categories.

Citation: Zeledón JG, Kaiser M, Spring O (2016) An Extended Leaf Disc Test for Virulence Assessment in *Plasmopara viticola* and Detection of Downy Mildew Resistance in *Vitis*. J Plant Pathol Microbiol 7: 353. doi:10.4172/2157-7471.1000353
the reaction found on *V. coignetiae*. On the contrary, the five strains produced a strong sporulation similar to *Vitis riparia* sporulation, single sporangiophores, with necrosis absent or weak. In *V. aestivalis* rather a moderate to strong necrosis with each of the five strains. *V. vinifera* showed no sporulation, but some sporulation on this host. It was observed that hyphae in the necrotic area possessed haustoria, thus gaining access to the host’s nutritional resources. Similar observations were made on the leaves of *A. japonica*. Although this host presented no infection symptoms at all, early developing intercellular structures of *P. viticola* were found at 72 hpi.

The leaves of *V. jacquemontii* showed the highest density of trichomes of all the tested species. The inoculum drops, when applied in the usual manner of the bioassay, were often not able to reach the leaf surface and the stomata for penetration. In some cases, however, sporulation was found on this host (Figure 4a), but the repeatability was very low and the sporulation was difficult to observe between the whiter felt of trichomes. For this reason, it was decided not to include this species in the phenotypic characterization system. Nevertheless, the microscopic analysis showed that *V. jacquemontii* is not completely resistant to downy mildew. The dense layer of trichomes (Figure 4b) impeded the observation of the infection process and the hyphal growth from the lower leaf side, but when clarified leaves were treated with aniline blue and observed from the upper side, hyphae with haustoria occasionally became visible. Empty sporangia and encysted spores were found in the inoculated area. In some cases, encysted spores were observed germinating into the stomata (Figure 4c). In addition, the microscopic analysis of the upper leaf surface revealed the presence of a high number of crystals in raphide bundles and druses deposited on the leaves of this species (Figure 4d).

**Discussion**

**The characterization system**

The ability to characterize *P. viticola* strains represents an important step towards improved breeding strategies. In our previously published study [7], a set of six host genotypes (two North American species, one European and three commercially used cultivars) was developed to achieve an improved assessment of strain diversity of the oomycete. In the present study, we broaden the characterization system by including

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**Table 1: Virulence assessment of five selected *Plasmopara viticola* strains on leaf discs of different hosts according to the new proposed system considering sporulation and necrosis separately.**

| Strains   | Group 1: *V. vinifera* | Group 2: North America | Group 3: Asia |
|-----------|------------------------|------------------------|--------------|
|           | MT | REG | CAB | SYL | RUP | RIP | CIN | AES | COI | AME | BET | AMP |
| 1117-A21  | ++ | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
| 1135-F2   | ++ | -   | -   | -   | +   | +   | -   | -   | -   | -   | -   | -   |
| 1136-A15  | +++| +   | +   | +   | +   | +   | -   | -   | -   | -   | -   | -   |
| 1137-C20  | +   | +   | +   | -   | +   | -   | +   | +   | +   | -   | -   | -   |
| 1191-B11  | ++ | -   | -   | -   | +   | +   | -   | -   | +   | -   | -   | -   |

**Figure 2: Virulence assessment of five selected *Plasmopara viticola* strains on leaf discs of different hosts according to the new proposed system considering sporulation and necrosis separately.**

Absence of sporulation

While *V. vinifera* cv. Müller-Thurgau showed a strong sporulation of 10 dpi, the leaf discs of *V. amurensis* showed a strong necrotic reaction without sporulation. This implicated an early interruption of the infection process before the pathogen was able to establish in the host tissue. However, microscopic analysis rejected this assumption. As shown in Figure 3, the pathogen penetrated the plant intercellular system and established a mycelium, but grew slowly and did not show sporulation on this host. It was observed that hyphae in the necrotic area possessed haustoria, thus gaining access to the host’s nutritional resources. Similar observations were made on the leaves of *A. japonica*. Although this host presented no infection symptoms at all, early developing intercellular structures of *P. viticola* were found at 72 hpi.

**Figure 3: Fluorescence micrographs of (a) *Vitis vinifera* cv. Müller-Thurgau, (b) *Vitis amurensis* and (c) *Ampelopsis japonica* infected leaves 72 hpi.** A highly virulent strain of *Plasmopara viticola* was used for inoculation (1137-C20). Symptoms at 10 dpi. Arrow heads indicate the haustoria. Aniline blue was used for staining.
Another limiting issue when establishing such a characterization system is the challenge of the exact definition of the host genotypes used as differentials. This point is especially difficult in the case of the grapevine cultivars, where many different crosses have been performed over the years, even to a point in which pedigree determination becomes very difficult, e.g. Regent [20]. This point also becomes important in the case of wild species when considering the possibility of hybridization with other species such as V. vinifera ssp. sylvestris, which may be naturally found close to grape-growing regions [21].

**North American and Asiatic species**

The North American Vitis species are known to be highly resistant to *P. viticola*. This oomycete native of North America has been present throughout the evolutionary history of the *Vitis* species in that region, exerting a selective pressure to develop resistance mechanisms against the pathogen. Nevertheless, just one species, namely *V. riparia*, was able to completely block the sporulation of the selected strains. Several loci related to resistance have been reported to have originated from this species [22,23]. However, sporulation in *V. riparia* has already been reported by other authors [24], showing that the reaction of this species to the pathogen may vary depending on the accession used [25]. Inoculum concentration, sporangia viability and environmental conditions might play an important role in the sporulation response of this highly resistant species.

In contrast to previously published results [26], sporulation was found in all the tested strains on *V. rupestris* and we were never able to detect sterile hyphae emerging from the stomata, as reported by those authors. *Vitis cinerea* and *V. aestivalis* had been reported as moderately resistant against downy mildew [25,27], however the fact that moderate to strong sporulation was found on these species is an indicator of downy mildew strain-specific reactions [27-29].

Asiatic *Vitis* species are known as important sources of resistance against *P. viticola* [30-32]. Thirty-five of the more than sixty known species from the genus *Vitis*, originate in China and many of these wild grapes have been used for wine production recently due to their desirable characteristics [30], which confirms their potential for breeding with European species. Even though the presence of several resistant loci have been reported on *V. amurenensis* (Rpv6: [14]; Rpv10: [31]; Rpv12: [33]), it has been shown that there is a strong variation of downy mildew resistance between accessions of this species. While some accessions present a high level of resistance to the pathogen [34], other accessions are susceptible to it [30,35]. This underlines the importance of a careful selection of wild species accessions for phenotyping. A strong necrotic reaction with no sporulation on *V. amurenensis* showed a high level of resistance on the tested genotype in the present study. This Asiatic species has been previously characterized as suppressing sporulation while reacting with strong necrosis [4,33]. Despite this, it has been reported that stronger necrotic reaction correlates to higher susceptibility in crosses of *V. vinifera* with *V. amurenensis* [14]. This shows that hosts with stronger resistance might block the sporulation of the pathogen without showing necrosis, which was the case for *A. japonica* in the present study. No reports were found in the literature about this species, which was the only Asiatic species where no infection symptoms were found. This kind of reaction, which would correspond to a nonhost resistance, is more durable than the one conferred by R genes. Type I nonhost resistance (preformed plant defense mechanisms) would be ideal for breeding because it does not involve a hypersensitive reaction [36]. These results are of special interest, since species closely related to *V. vinifera* constitute potential sources of resistance genes against downy mildew e.g. *Muscardinia rotundifolia* [37].

Interestingly, the biggest differences between strains were found in species from different geographical regions. This enabled us to improve the resolution for screening *P. viticola* strains in terms of their ability to overcome many different defense mechanisms. A wider range of reactions can be characterized since the host genotypes range from fully susceptible to fully resistant.

The development of necrosis on a specific host reveals the presence of resistance genes [12-14] and the level of sporulation achieved by a strain is related to its aggressiveness [15]. Conventional evaluation systems used on *P. viticola* based on the amount of sporangia do not consider the necrotic reaction of the plant [16-18] or evaluate it in combination with the sporulation [13,19]. Our modified system considers both reactions: that of the plant (necrosis) and that of the pathogen (sporulation) separately, thereby facilitating the interpretation of the results.

The characterization of *P. viticola* strains poses some problems that are not present in the case of other oomycetes. In contrast to Plasmopara halstedii (downy mildew of sunflower) or Bremia lactucae (downy mildew of lettuce), the availability for year round cultivation of *P. viticola* is limited due to the perennial nature of its host. The possibility to cultivate the plants in the laboratory in a short period of time (in the case of sunflower or lettuce) facilitates the evaluation of strains even during winter. On the other hand, the perennial nature of *Vitis* imposes an advantage in terms of the homogeneity of the host material used for strain characterization. The possibility of a clonal propagation of the differential hosts assures a better repeatability of the test for other research groups compared to sunflower or lettuce. In those cases, the propagation occurs by means of seeds, thus demanding a higher number of replicates to obtain reliable results.
the analysis of the *V. vinifera* cultivars. In contrast to the Asiatic species where a very homogeneous response was displayed, the five strains showed extremely varied responses to the cultivars (e.g. Cabernet Cortis and Regent, Figure 2). The combination of resistance genes in the breeding process of the cultivars could be responsible for these results. The characterization system allowed us to detect interesting reactions such as that found when infecting the cultivar Regent with the strain 1137-C20 [38]. This strain, particular due to its strong sporulation on *V. coignetiae*, demonstrating that other mechanisms are responsible for resistance in the Asiatic species. A strain-specific reaction could explain this case, as mentioned before. The combination of strains with differing degrees of virulence in hosts with different resistance levels enables the study of genes responsible for a higher virulence or for the breakdown of resistance [11,39]. The possibility to study those mechanisms improves the understanding of the evolving capacity of European *P. viticola* strains [16], which provides valuable information that should be integrated in breeding programs.

**Absence of sporulation**

Microscopy showed similar results to those reported in the literature, where shortly after penetration into the substomatal cavity, resistant hosts were able to inhibit the pathogen development [24,26,40]. The response of *A. japonica* to the infection was similar to that reported on *M. rotundifolia* [24]. In both cases, a very low number of infection structures were found and the growth was hindered shortly after the formation of the first haustoria. The absence of necrosis on the inoculated leaves of *A. japonica* deserves further investigation. A very efficient response may have caused this kind of reaction [24] which could be of interest for breeders.

The reduced capacity of *P. viticola* to infect the Asiatic species *V. jacquemontii* is attributable to a mechanical barrier imposed by the dense coverage of trichomes found on the lower side of the leaves. This barrier impedes the direct contact of the zoospores with the stomata. The leaf hairiness of some wild *Vitis* species has been reported as an important factor for impeding an infection of downy mildew [25]. 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 contradicts previous reports [40] where the formation of long surface mycelia is described. We never observed long external mycelia, but rather a very low number of encysted spores with normal germ tubes entering into the stomata and forming normal hyphae with haustoria.

Although numerous studies [12,24,34,40,41] have been conducted on the interaction between *P. viticola* and *Vitis* species and cultivars in the last years, still many questions remain unanswered. Which factors are able to hinder the hyphal growth on the resistant species? How do resistance genes exert their activity? Is it one gene or is it a combination of many genes that confer resistance? Which factors enable *P. viticola* strains to overcome the defense reaction? The virulence assessment described here will help to select suitable host-pathogen combinations which will help to address these questions and to unravel the mechanisms behind this complex interaction.

**Acknowledgment**

This work was partially financed by a scholarship from the German Academic Exchange Service (DAAD) for Javier Gómez-Zeledón. We are thankful to Peter Nick and Viktoria Trössler from the Karlsruhe Institute of Technology for providing most of the plant material used in this study. We would also like to thank Nikolaus Merkt for his support with plant cultivation and to Margaret Janke for proofreading the manuscript.

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