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. rupestris 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 inconsistent 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...
Asian wild species in order to broaden the basis of genetically differing resistance mechanisms.

Material and Methods

The characterization system

For the phenotypic characterization of V. 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: Pfaffnweiler, Germany/Regent, 1137-C20: Pfaffnweiler, Germany/Gutedel, 1191-B11: Laufen, Germany/Lemberger. These strains were selected based on their characteristic reaction on six different grapevine genotypes of three V. vinifera cultivars and three wild Vitis species [7]. The pathogens were subcultured in the laboratory using V. vinifera leaves from the cultivars Müller-Thurgau and Bacchus. The subculture and handling of the pathogen were performed following the previously published methodology. The characterization system incorporated V. vinifera cultivars with different levels of resistance and wild Vitis species. To incorporate additional interesting infection reactions, Asiatic and North American species from the Botanical Garden, Karlsruhe Institute of Technology (KIT) were selected (for identification see ID numbers below). The genotypes were divided in three groups. The first group consisted of V. vinifera genotypes: cv. Müller Thurgau (ID: FR3 vg); cv. Regent (ID: rpv.3); cv. Cabernet Cortis (ID: FR680) and V. vinifera ssp. sylvestris. In the second group, North American grapevine species were considered: Vitis riparia (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: 5644). The reaction of V. jacquemontii (ID: 5883) was analyzed 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

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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 (+).
necrotic reaction on *V. amurensis* showed that different physiological processes are involved in the interaction.

Amongst the tested strains, all five samples could be differentiated by their sporulation performance on the 12 hosts. Moreover, some strains behaved quite exceptionally in specific combinations, which could make them interesting for further studies on molecular mechanisms behind compatible or incompatible reactions. For instance, strain 1135-F2 did not sporulate on Cabernet Cortis, although it did not behave generally less aggressive on other hosts. An interesting infection reaction was also observed with strain 1137-C20 when inoculated on Regent. Although the other strains were barely able to sporulate on this cultivar, 1137-C20 achieved an unrestricted sporulation such as seen on the generally susceptible cultivar Müller-Thurgau. Further studies will be conducted on this host-pathogen combination to determine the molecular mechanisms responsible for the breakdown of the resistance.

**North American and Asiatic species**

In contrast to the cultivars of *V. vinifera*, six of the eight Asiatic and North American wild species did not allow differentiation between the five strains with respect to sporulation intensity. Only three cases in *V. rupestris* and one case in *V. coignetiae* showed differences (Figure 2). On the other hand, susceptibility against *P. viticola* in general varied considerably between the host genotypes. Only one out of the four selected Asiatic species, namely *A. japonica*, showed no symptoms at all when inoculated with any of the five strains. In contrast, all five strains achieved strong sporulation in *V. betulifolia*, similar or even stronger than that achieved in the control *V. vinifera* cv. Müller-Thurgau. In *V. coignetiae*, the four of the five strains were able to strongly sporulate while 1137-C20 showed only moderate sporulation. Infections of this species were characterized by the absence of necrosis which contrasts to the reaction of *V. amurensis*. The latter showed no sporulation, but rather a moderate to strong necrosis with each of the five strains.

In regards to the North American species, *V. riparia* was confirmed as the most resistant species followed by *V. aestivalis* and *V. rupestris*. In *V. aestivalis*, the highest infection category achieved was D (weak sporulation, single sporangiophores), with necrosis absent or weak. *Vitis riparia* allowed no sporulation and seldom showed necrotic spots. On *V. cinerea*, the five strains produced a strong sporulation similar to the reaction found on *V. coignetiae*.

**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.

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 whitish 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...
system is the challenge of the exact definition of the host genotypes.

Those cases, the propagation occurs by means of seeds, thus demanding the test for other research groups compared to sunflower or lettuce. In the case of sunflower or lettuce, the perennial nature of its host 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.

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. amurensis (Rpv8: [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. amurensis 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. amurensis [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. Muscadinia rotundifolia [37].

Interestingly, the biggest differences between strains were found in...
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 infected 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 impeded 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.

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