Disease Control and Integrated Management

The Characterization of Pathotypes in Grapevine Downy Mildew Provides Insights into the Breakdown of Rpv3, Rpv10, and Rpv12 Factors in Grapevines

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Abstract

We describe a standard method for characterizing the virulence profile of *Plasmopara viticola*, the causal agent of grapevine downy mildew. We used 33 European strains to inoculate six grapevine varieties carrying the principal factors for resistance to downy mildew (Rpv1, Rpv3.1, Rpv3.2, Rpv5, Rpv6, Rpv10, and Rpv12) and the susceptible *Vitis vinifera* 'Chardonnay'. For each interaction, we characterized the level of sporulation by image analysis and the intensity of the grapevine hypersensitive response by visual score. We propose a definition for the breakdown of grapevine quantitative resistances combining these two traits. Among the 33 strains analyzed, 28 are virulent on at least one resistance factor. We identified five different pathotypes across the 33 strains analyzed: two pathotypes overcoming a single resistance factor (vir3.1 and vir3.2) and three complex pathotypes overcoming multiple resistance factors (vir3.1,3.2; vir3.2,12; vir3.1,3.2,10). Our findings confirm the widespread occurrence of *P. viticola* strains overcoming the Rpv3 haplotypes (28 strains). We also detected the first breakdown of resistance to the Rpv10 by a strain from Germany and the breakdown of Rpv12 factors by a strain from Hungary. The pathotyping method proposed here and the associated differential host range lay the groundwork for the early detection of resistance breakdown in grapevines. This approach will also facilitate the monitoring of the evolution of *P. viticola* populations at large spatial scales. This is an essential step forward to promoting durable management of the resistant grapevine varieties currently available.

Keywords: pathotype, plant−pathogen interaction, *Plasmopara viticola*, quantitative resistance, resistance breakdown, resistance durability

Grapevine downy mildew, caused by the obligate biotrophic oomycete *Plasmopara viticola* (Berk. & M. A. Curt.) Berl. & De Toni, is one of the most destructive oomycetes worldwide (Kamoun et al. 2015). *P. viticola* is native to North America, where it infects a large number of wild *Vitis* species (Rouxel et al. 2014, 2013). Following its initial introduction into European vineyards in the 1870s (Fontaine et al. 2013; Millardet 1881), it spread to all major grape-producing regions of the world (Fontaine et al. 2021). The Eurasian wine grape *Vitis vinifera* is highly sensitive to downy mildew and the control of this disease is currently largely based on fungicides. Resistance factors from American and Asian *Vitis* species conferring resistance to downy mildew, and known as Rpv for resistance to *P. viticola*, are currently being used to breed new disease-resistant varieties. More than 30 genetic factors conferring resistance to downy mildew have been identified (Maul 2021), but only a small number of these factors are currently used in European breeding programs. The so-called Rpv factors are encoded by major quantitative trait loci located in genomic regions rich in nucleotide-binding site-leucine-rich repeat-like resistance genes (Di Gaspero et al. 2012; Moroldo et al. 2008). These major resistances display monogenic inheritance, but are phenotypically quantitative (or partial), i.e., *P. viticola* strains develop on these varieties, but to a lesser extent than on wild-type varieties. The most widely used resistance factor is Rpv3, which was selected from the species *V. rupestris* (Bellin et al. 2009; Di Gaspero et al. 2012; Foria et al. 2020). The two major haplotypes used in breeding programs are Rpv3.1 (Rpv3[299-279]) identified in 'Seibel 4614' and Rpv3.2 (Rpv3[null-279]) identified in 'Munson' ('Jaeger 70'). The other major resistance factors currently used in breeding programs are Rpv1 (Merdinoglu et al. 2003), from *Muscadinia rotundifolia*, Rpv10 (Schwander et al. 2012), from *V. amurensis*, and Rpv12 (Venuti et al. 2013), also from *V. amurensis*. As in many perennial crops, concerns about the durability of these grapevine resistance factors are magnified by the long duration of breeding schemes (16 to 17 years [Merdinoglu et al. 2018]) and the lifespan of the plant (about 20 to 30 years).

Due to its large population size and its capacity for sexual reproduction (Gessler et al. 2011), *P. viticola* has a high evolutionary potential, as illustrated by its rapid adaptation to synthetic fungicides (Blum et al. 2010; Chen et al. 2007; Delmas et al. 2016). The breakdown of the Rpv3.1 factor present in 'Bianca' and 'Regent' is another example of the rapid adaptation of *P. viticola* to its host (Delmotte et al. 2014; Heyman et al. 2021; Peressotti et al. 2010). Indeed, in this context, virulence emerged within 5 years on at least three independent occasions, in three different wine-producing areas (Delmotte et al. 2014). Recently, Wingerter et al. (2021) reported the discovery of a *P. viticola* isolate able to overcome both Rpv3.1 and Rpv12 factors. Using a larger host range including 'Bronner' and 'Prior', Delmas et al. (2016) reported an increase of the sporulation level of *P. viticola* strains on resistant varieties carrying the Rpv10

factor. Gómez-Zeledón et al. (2017) and Heyman et al. (2021) also described three strains that were able to sporulate strongly on varieties carrying the Rpv10 factor, but without abolishing the hypersensitive response (HR) of the plants.

The isolate-specific behavior of *P. viticola* on resistant grapevine varieties strongly suggests a gene-for-gene interaction, highlighting the need for a system of pathotype characterization to describe these interactions. This approach is commonly used in crops where breeders had selected cultivars carrying qualitative resistance to pathogens (Black et al. 1953; Gulya et al. 1998; Johnson et al. 1972; Van Ettekoven and Van der Arend 1999). However, for host−pathogen interactions characterized by phenotypically quantitative responses to disease, as for *V. vinifera*−*P. viticola* interaction, the identification of a strain that breaks resistance is not straightforward. A methodology based on analyses of the quantitative response of the pathogen is therefore required to define the breakdown of resistance in this context. The current reference method for measuring the leaf resistance of a grapevine variety to *P. viticola* is the OIV-452 descriptor (Anonymous 1983) adapted by Bellin et al. (2009) for laboratory bioassays. This rating scale combines visual assessments of sporulation and HR in a single score. Gómez-Zeledón et al. (2013, 2017) took a step forward by proposing a symptom rating scale for characterizing the phenotype of five *P. viticola* strains on six wild *Vitis* species and three resistant varieties. This constituted a major step towards a pathotype characterization method, although the differential host used did not cover the range of resistance factors currently present in resistant grapevine varieties. More recently, using a scoring system adapted from previous studies (Gómez-Zeledón et al. 2016; Schwander et al. 2012), Heyman et al. (2021) assessed the development of five *P. viticola* strains on a range of 16 resistant grapevine varieties carrying multiple combinations of Rpv loci. The five isolates displayed considerable phenotypic variability when used to inoculate multiple resistant hosts carrying various resistance factors. Improvements in the definition of grapevine downy mildew pathotypes are therefore required to take into account the variability of this pathogen, which has been little considered to date.

In this study, we propose a methodology for defining the breakdown of resistance within the particular context of phenotypically quantitative resistance. We used a collection of 33 *P. viticola* strains to inoculate six differential hosts carrying the main Rpv factors released in Europe in resistant varieties and the susceptible *Vitis vinifera* 'Chardonnay'. Pathogen development and plant reaction were assessed by rating pathogen sporulation and the degree of HR due to the effector-triggered immunity of the plant. We were able to detect the breakdown of resistance for four quantitative resistance factors and to describe five different pathotypes. These results are discussed with a view to guiding the worldwide deployment of resistant grapevine varieties.

Materials and Methods

Plant and pathogen material and isolation of monosporangia

We selected six grapevine varieties representing the most of the partial resistance factors to *P. viticola* used in European breeding programs (Table 1). Most resistance factors are present in a single variety. We used the two main haplotypes of the Rpv3 resistance:

Rpv3.1 and Rpv3.2, which we considered as two distinct resistance factors. The Rpv3.1 locus is incorporated in 'Regent', which is descended from 'Seibel 4614' (Maul 2021) and which also carries the minor factors Rpv11 and Rpv4 (Fischer et al. 2004; Welter et al. 2007). The Rpv3.2 locus is present in 'Seibel2', which is descended from 'Munson' ('Jaeger 70') (Di Gaspero et al. 2012). The Rpv5 and Rpv6 loci are present in the 'Riparia Gloire de Montpellier' (RGM) rootstock (Marguerit et al. 2009). The French variety 3160-12-3N, carrying Rpv1 (Merdinoglu et al. 2003), has yet to be released onto the market, and is currently undergoing testing in an experimental vineyard. 'Solaris' is the genotype of origin of the major factor Rpv10 (Schwander et al. 2012), but it also carries the minor factor Rpv11 (Schwander et al. 2012) and Rpv3.3 (Di Gaspero et al. 2012). Finally, 'Kunleany' carries the Rpv12 factor in an imprecise genetic background (Venuti et al. 2013). The widely distributed *V. vinifera* L. 'Chardonnay' was included as the susceptible reference cultivar in the experiment. Budwood cuttings of Chardonnay, Regent, and Solaris were obtained from the INRAE experimental vineyard in Bordeaux but are also available in nurseries, cuttings of Seibel2, Kunleany, and RGM were obtained from the French ampelographic collection at Vassal-Montpellier (note that RGM is also available in nurseries), and cuttings of 3160-12-3N were obtained from the INRAE experimental vineyard in Pech-Rouge. These cuttings were grown simultaneously in a greenhouse under natural photoperiod conditions, without chemical treatment. The cross-inoculation experiment was conducted on leaves collected after 3 months of cultivation.

Isolates were collected between 2010 and 2016 from resistant and susceptible grapevines in France ($n = 9$), Italy ($n = 5$), Germany ($n = 4$), Spain ($n = 4$), Switzerland ($n = 3$), Hungary ($n = 3$), the Czech Republic ($n = 2$), Bulgaria ($n = 1$), Georgia ($n = 1$) and Lebanon ($n = 1$) (Supplementary Table S1). Each isolate consisted of a single sporulating lesion collected from a single infected grape leaf. The leaf fragments were rinsed with sterile water and left overnight in the dark to allow sporulation to occur. Fresh sporangia were collected and stored in liquid nitrogen for subsequent experiments. For each isolate, the sporulating leaf fragments stored in liquid nitrogen were gently agitated against a microscope slide to release the sporangia. Under a binocular microscope, a single sporangium was caught with a disinfected human eyelash and gently deposited on a 15 µl droplet of reverse-osmosis water at the center of a 15-mm-diameter leaf disc cut from a *V. vinifera* 'Cabernet Sauvignon' plant. The inoculated discs were placed overnight in the dark. The water droplets were removed by suction and then the discs were incubated for 6 days at 23°C, under a 12-h light/12-h dark photoperiod. The infection efficiency of a single sporangium is low (about 10%). We therefore isolated several sporangia in this way. After 6 days of incubation in a growth chamber, the infected leaf discs (one disc per isolate) were placed in Eppendorf tubes and left overnight in a desiccator before storage at −20°C. The isolates obtained by monosporangium isolation are referred to hereafter as strains.

Two weeks before the experiment, the strains were propagated on five different leaves of Cabernet Sauvignon. After 1 week of incubation, they were then propagated on four detached leaves for the cross-inoculation experiment. One day before the experiment, the sporulating leaves were gently rinsed with distilled water to remove the sporangia already present, to ensure the collection of fresh

TABLE 1. Characteristics of the grapevine varieties used for the pathotyping experiment

Host plant	Pedigree	Major resistance factor
Chardonnay	−	None
3160-12-3N	BC4 of *Vitis vinifera* × *Muscadinia rotundifolia*	Rpv1
Regent	Diana × Chambourcin	Rpv3.1
Seibel2	Jaeger 70 × *V. vinifera*	Rpv3.2
Riparia Gloire de Montpellier	*V. riparia* Michaux	Rpv5, Rpv6
Solaris	Merzling × Geisenheim 6493	Rpv10
Kunleany	(*V. amurensis* × *V. vinifera*) × Afus Ali	Rpv12

sporangia of the same age on the following day. On the day of inoculation, the strains were suspended in sterile water and the density of the suspension was adjusted to 10^5 sporangia ml^{-1} with a portable particle counter (Scepter 2.0 automated cell counter; Millipore).

Cross-inoculation experiment

We analyzed 231 plant−pathogen interactions (33 strains × 7 varieties), using five replicates per interaction, for a total of 1,155 samples. We also performed three mock inoculations, by inoculating water on each host, as a negative control (total of 105 discs). We therefore inoculated 1260 samples in total. Inoculations were performed on the fourth leaf below the apex. Leaves were washed with distilled water and dried on absorbent paper. We excised leaf discs with a diameter of 15 mm with a cork borer and placed them, abaxial side up, on wet filter paper in a Petri dish. For a given interaction, each of the five individual leaf discs was collected from a different plant (variable *IdP* in the statistical analysis, see below). For each of the 33 strains, each of the 35 discs (= five replicates × seven varieties) was sprayed with 4 ml of downy mildew suspension. We allowed the surface of the leaf discs to dry overnight, to prevent the development of mold and bacteria. The discs were then placed in 15 square Petri dishes (23 × 23 cm). The 15 Petri dishes were organized in three batches of five dishes containing 11 strains and one control each. This permits to distribute the five replicates among dishes. We took care that the plates containing the same strains were not on the same location, location being defined here as the combination of growth chamber × shelves (*LMS* in the statistical analysis, see below). Petri dishes were sealed with Parafilm to maintain relative humidity at 100%. The leaf discs incubated for 6 days at 18°C, to keep them alive for the duration of the incubation without impacting the sporulation, under a 12-h light/12-h dark photoperiod.

Evaluation of sporulation and HR

Sporulation and HR intensities were measured at 6 days postinoculation on the 1,260 leaf discs inoculated. The OIV-452-1 descriptor was initially proposed by the OIV (Organisation Internationale de la Vigne et du Vin) (Anonymous 1983) and was used as adapted by Bellin et al. (2009) to evaluate the degree of resistance of the grapevine to downy mildew on leaf discs (Fig. 1). This variable is referred to hereafter as *OIV*.

For sporulation intensity, a visual sporulation scores of 0 (no sporulation observed) to 5 (dense sporulation) was attributed (Fig. 1). This variable is referred to hereafter as *SPO*.

Sporangium production was assessed by determining the number of sporangia per mm² on each disc with a Multisizer 3 automatic particle counter (Coulter Counter Multisizer 3; Beckman Coulter). Leaf discs were placed separately in 10 ml of saline solution (Isoton II, Beckman Coulter) and processed as previously described (Delmas et al. 2014). Briefly, particles suspended in the saline solution are drawn through a small aperture (100 μm) separating two electrodes and displaced their own volume of electrolyte, which increase the impedance of the aperture momentarily. For each particle, the analyzer Multisizer 3 calculates a volume based on the extent of the change in impedance, and thus measure the size of the particle. We counted the particles from 6 to 20 μm in diameter and thus obtained a number of particles per discs that we conversed into a number of particles per square millimeter. This variable is referred to hereafter as *SpNb*.

The sporulation area was determined by image analysis. We took 25 photographs of each square Petri dish with a Canon EOS 650D camera equipped with a macro lens (Canon EF 100 mm f/2.8 USM). Photographs were taken in manual mode (f/5.6; ISO-100). Each 17.9-megapixel image, containing four (or sometimes three) individual leaf discs, was analyzed with ImageJ (version 1.52a) and a dedicated plugin described at and available from GitHub: https://github.com/ManonPaineau/image_analysis_P.viticola. Briefly, the plugin performed two main steps for the analysis of each

four-leaf disc image: disc identification and the evaluation of sporulation. In the first step, the colored image (RGB) is saturated and then transformed into a binary image. The largest pixel sets are identified as the discs of interest. These pixel sets are applied to the original RGB image, identifying the discs according to their positions in the image. An individual image is then recorded for each leaf disc. In our experimental conditions, a leaf disc corresponded to a mean of 1.48 megapixels (SD = 35,807 pixels). Each pixel set (i.e., leaf disc) was then analyzed in the second step. The saturation threshold was adjusted to focus exclusively on current sporulation. Both the original and saturated images were displayed on the screen to facilitate this step. Once the threshold had been set, the number of black pixels (corresponding to sporulation) for each disc was determined automatically. The plugin saved output images at each step, to facilitate subsequent verification. We calculated the sporulation area as a percentage, by dividing the number of black pixels by the total number of pixels and then multiplying by 100. This variable is referred to hereafter as *SpPr*.

The necrosis pattern (NP) is the shape, color, and size of the necrosis, and is described by a qualitative score (Supplementary Fig. S1). We focused on necroses resulting from the HR (NP score of 5, 7, or 9) for further analyses. HR intensity was analyzed with a visual score, ranging from 0 to 4, based on the number of necroses resulting from HR per leaf disk, as follows: 0 = no necroses; 1 = <10 necroses; 2 = from 10 to 30 necroses; 3 = from 30 to 60 necroses; and 4 = >60 necroses (Fig. 1). This variable is referred to hereafter as *HR*.

Statistical analysis

We first rated each leaf disc with four visual scores: i) sporulation intensity $SPO_{v,i,r}$ indexed by host variety v ($1 \leq v \leq 7$), strain i ($1 \leq i \leq 33$), and biological replicate r ($1 \leq r \leq 5$): (ii) HR intensity $HR_{v,i,r}$, (iii) the necrosis pattern $Np_{v,i,r}$, and (iv) the OIV score $OIV_{v,i,r}$. Image analysis and a particle counter were then used to measure sporulation. Image analysis was used to estimate the proportion of the area displaying sporulation $SpPr_{v,i,r}$ as the ratio of the number of sporulating pixels $NPS_{v,i,r}$ to the total number of pixels $NPT_{v,i,r}$. Similarly, we used $SpNb_{v,i,r}$ to denote the number of sporangia per square millimeter as determined by the particle counter. The corrected variables $NPSc_{v,i,r}$, $SpPrc_{v,i,r}$, and $SpNbc_{v,i,r}$ were obtained by setting the value to 0 for all leaf discs without sporulation visible by eye (i.e., such that $SPO_{v,i,r} = 0$). In addition to these response variables, the experimental design involved the following explanatory variables: (i) the inoculated host plant *InoH* (7 levels), (ii) the pathogen strain *ISO* (33 levels), (iii) the growth chamber *LMS* (4 levels, two growth chambers times two shelves), and (iv) the individual plant from which leaf discs were cut *IdP*. Leaf discs were cut from a total of 104 plants, with 1 to 18 leaf discs obtained from each plant (mean = 12.7, SD = 5.6). We performed the statistical analysis on 1,155 samples as mock strains were not included in the analysis.

We first investigated the relationship between the main traits of sporulation measured. In particular, we explored the relationship between *SpNb* (response variable) and *SpPr* (explanatory variable) by fitting a generalized linear model (GLM) with a quasi-Poisson distribution.

We then evaluated the effect of the inoculated host *InoH* on the intensities of HR and sporulation, by considering the 1153 out of 1,155 leaf discs for which image analysis data were available. We assessed this effect using generalized linear mixed models (GLMMs) to take into account the pseudoreplication caused by the hierarchical nature of the cross-inoculation experiment: (i) *IdP* was considered as a random intercept effect, to take into account the excision of several leaf discs from the same plant and (ii) *ISO* was also considered as a random intercept effect, to take into account the inoculation of several discs with the same strain. Specifically, the effects of *InoH* (fixed effect), *LMS* (fixed effect), *ISO*, and *IdP* on the qualitative ordered response variable *HR* were analyzed with cumulative link

mixed models (CLMM). We also analyzed the same four effects plus *HR* (fixed effect) on the zero-inflated binomial (ZIB) response variable $NPSc_{v,i,r}$. A ZIB distribution was used to account for the lack of infection in 141 of the 1,153 discs analyzed. The zero-inflated part of the model considered only the explanatory variable *HR*. The models are detailed in Supplementary Table S3 (ZIB) and Supplementary Table S4 (CLMM).

We then visualized the 231 plant−pathogen interactions (33 strains × 7 grapevines varieties) via a genotype−genotype interaction matrix. This interaction matrix was plotted as a heatmap, by applying the "complete" clustering technique to the variable *SpPrc*. HR data were inserted into the matrix for the simultaneous visualization of both sporulation and HR data. We then used the Kendall correlation coefficient to calculate the correlation matrix
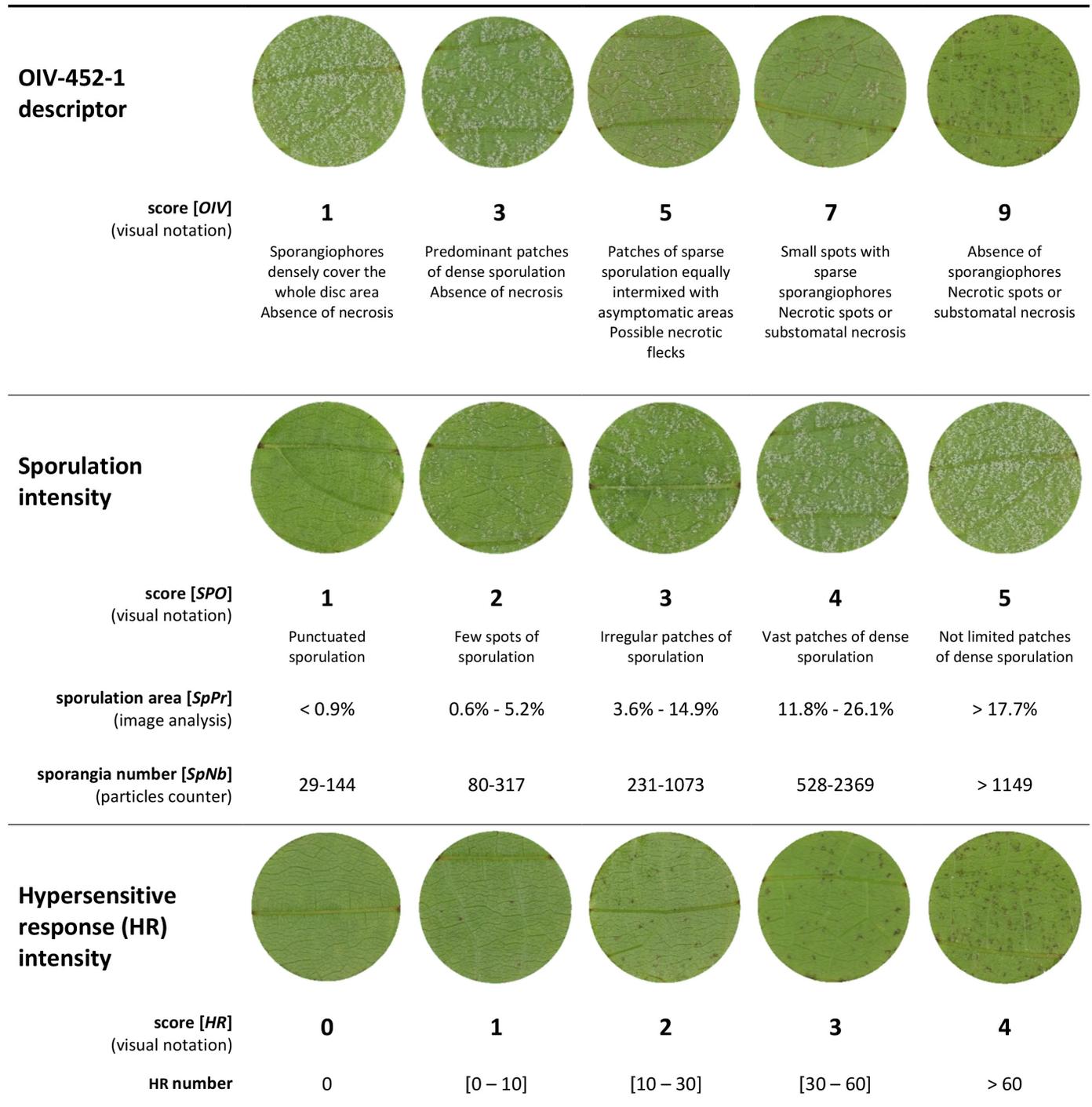
Fig. 1. Scale for scoring downy mildew symptoms on grapevine leaf discs. The evaluation was performed 6 days postinoculation on the abaxial side of the leaf disc. The OIV-452-1 descriptor is adapted from Bellin et al. (2009). Sporulation intensity was assessed on the basis of (i) a visual score from 0 to 5 (although the score of 0, corresponding to no observed sporulation, is not represented), (ii) the range of sporulation area obtained on image analysis, and (iii) the range of the number of sporangia per square millimeter obtained with a particle counter. For each visual sporulation score, the ranges (5th and 95th percentiles) of the sporulation area and sporangium number measures are indicated (Supplementary Fig. S3). Note that the image analysis and particle counter data were unable to differentiate between a total absence of sporulation and a minimal level of sporulation. A visual assessment was required in such cases. The intensity of the hypersensitive response (HR) was assessed by assigning a visual score of 0 to 4 based on the number of HR observed.

between sporulation area *SpPrc* and HR intensity *HR* for all pairs of inoculated hosts.

Statistical analyses were performed with R software version 4.0 (R Core Team 2020). The CLMMs were fitted with the package ordinal (Christensen 2019). The GLMMs with the ZIB distribution were fitted with the glmmTMB package (Brooks et al. 2017) and the residuals were checked with the DHARMa package (Hartig and Lohse 2020). After GLMM analysis, a comparison of means was performed, using the marginal means estimated with the emmeans package (Lenth 2016). Compact display letters are used to indicate significant differences between means at $P < 0.05$ in Tukey's multiple comparison test. The heatmap and correlation matrix were visualized with the pheatmap (Kolde 2019) and corrplot (Wei 2021) packages, respectively. The dataset to reproduce the analysis is available in the Supplementary Materials.

Results

High correlation between three methods of sporulation measurements

We evaluated the intensity of *P. viticola* sporulation by (i) image analysis (*SpPr*), (ii) particle counting (*SpNb*), and (iii) visual observation (*SPO*) (Supplementary Fig. S2). The strong relationship between *SpPr* and *SpNb* revealed substantial overdispersion, as the variance of *SpNb* largely exceeded its mean (the dispersion parameter of the quasi-Poisson distribution is estimated at 121.7). The relationships between the visual score *SPO* (with six levels of sporulation, from 0 to 5) and the continuous estimates of sporulation *SpNb* and *SpPr* were explored through the Supplementary Figure S3. Both particles counter (Supplementary Fig. S3A) and image analysis (Supplementary Fig. S3B) weakly discriminated between leaf discs without sporulation (*SPO* = 0) and those with

punctate sporulation (*SPO* = 1). Better differentiation was clearly obtained when we considered only leaf discs with a *SPO* $\geq$ 1. Visual scores remained indispensable for detecting the presence of sporulation, but quantitative measurement methods for discs displaying sporulation were able to provide continuous estimates of sporulation intensity. The three methodologies tested to measure sporulation intensities produced highly correlated values for leaf discs displaying sporulation. Accordingly, we focus our analysis below on the estimation of sporulation area provided by image analysis.

High sporulation is not always associated with low HR

To evaluate the interest to measure sporulation and HR separately or to rate both traits at the same time as in the official *OIV*-452-1 score, we compared the *OIV* notation with both *SpPr* and *HR*. The relationships to each of these symptoms considered separately, sporulation area *SpPr* on the one hand, and the number of HR spots on the other, are displayed in Figure 2. A strong correlation coefficient was obtained for the relationship between *OIV* and *SpPr* ($r = -0.86$), a high OIV score being associated with low levels of sporulation (Fig. 2A). Conversely, a weak correlation was observed between *OIV* and *HR* ($r = 0.42$) (Fig. 2B). The relationship was dichotomous, with *OIV* $\leq$ 5 associated with low *HR* (scores $\leq$1) and *OIV* scores >5 being indistinctly associated with *HR* scores ranging from 1 to 4. Based on those results, the evaluation of both sporulation and HR separately seems more appropriate to describe the diversity of host−pathogen interactions.

Downy mildew symptoms vary according to the resistant plant inoculated

To analyze the effect of the seven grapevine varieties on the intensities of HR and sporulation induced by our set of the 33 Eurasian strains, we performed a cross-inoculation experiment. The CLMMs (Supplementary Table S4) and GLMMs (Supplementary
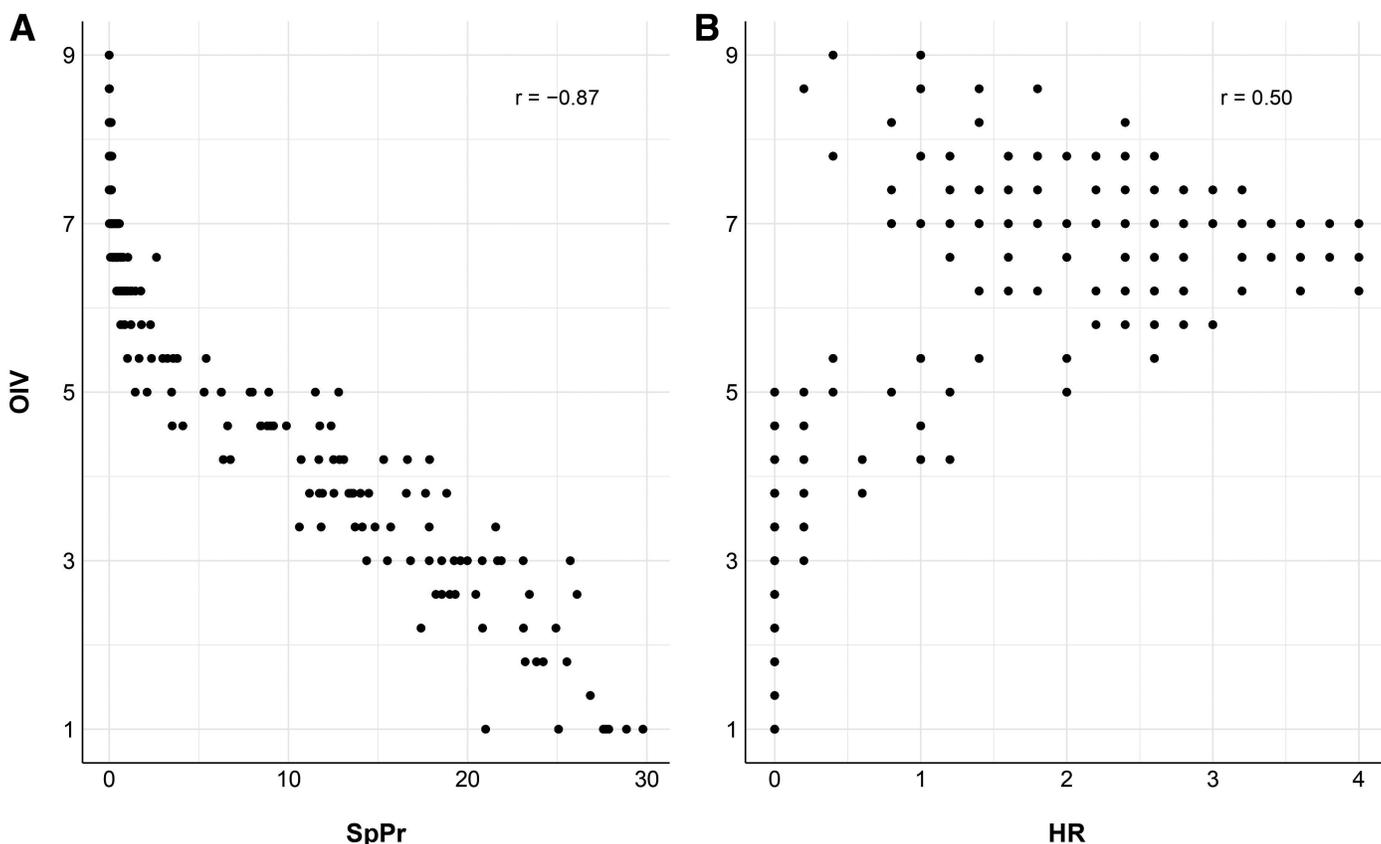
Fig. 2. Correlation between the OIV-452-1 score and the **A,** measured sporulation area and **B,** observed hypersensitive response (HR) score. The OIV-452-1 score has a minimum value of 0 and a maximum value of 9 and takes into account both sporulation and HR. The sporulation area is given as a percentage of area of the leaf disc displaying sporulation, as measured by image analysis, and the HR score is given as a score from 0 (no HR observed) to 4 (more than 60 HR spots observed per leaf disc). All points on both graphs represent overlays of several points of the same value. In total, 1,153 values are represented on both graphs.

Table S3) used to test this effect fitted the data in a satisfactory manner. The effect of *InoH* was highly significant for both HR (P value $< 10^{-6}$) and for sporulation area ($P < 10^{-6}$). Furthermore, the ZIB model used to analyze sporulation area demonstrated that (i) the probability of presence of sporulation on leaf discs decreased with increasing HR intensity (Supplementary Table S3) and (ii) for the 1,012 leaf discs presenting sporulation, the sporulation area decreased with HR intensity ($P < 10^{-6}$). The pairwise significant differences between the seven varieties for the intensities of HR and sporulation are shown in Figure 3.

The susceptible variety *V. vinifera* 'Chardonnay' was used as the baseline for assessments of the effect of resistant varieties. Chardonnay had a large sporulation area (mean 19.96%) and HR was absent from almost all the discs inoculated. A similar pattern was observed for Seibel2 (carrying the Rpv3.2 factor), with almost no HR and a lower sporulation area (mean 13.20%). Similarly, the variety carrying the Rpv3.1 factor, Regent, had high mean sporulation areas (8.22%) associated with a few necrotic lesions. Regent also displayed high variability for these two traits. The sporulation area and HR intensity profiles of the other four varieties (3160-12-3N, RGM, Solaris, and Kunleany) were different. They displayed highly effective resistance, as attested by their weak levels of sporulation (mean sporulation area ranging from 0.09 to 1.51%) and high levels of HR. Most developed many necrotic lesions (HR score >2), but RGM scores were close to 1 (fewer than 10 necrotic lesions observed per discs). Nevertheless, despite their high resistance levels, a few strains yielded sporulation areas greater than 6% on Solaris and Kunleany, as shown by the individual points on the Solaris and Kunleany boxplot, highlighting the importance of also studying the strains at individual level.

The GLMM analysis also revealed effects linked to the experimental design. In particular, the inclusion of a random intercept effect to control for several leaf discs being excised from the same plant (*IdP*) greatly improved the fit of the model for the intensities of both sporulation (Supplementary Table S3) and HR (Supplementary Table S4). The effect of growth chamber (*LMS*) was smaller, but nevertheless significant for the intensity of sporulation ($P < 10^{-6}$) but not for the intensity of *HR* ($P = 0.053$). To summarize, the sporulation of the population of 33 strains analyzed is strongly impacted by the grapevine varieties. Our analysis also highlights strong individual plant effects that deserves to be properly control in cross-inoculation experimental design.

Contrasted phenotypic responses among *P. viticola* strains

In order to analyze the 231 host−strain interactions at individual level, we realized a heatmap showing, for each interaction, the average of sporulation and HR value for the five replicates (Fig. 4). Two groups of varieties can be distinguished according to the complete classification based on the rows and columns of this heatmap.

The first group consists of *V. vinifera* 'Chardonnay' and 'Seibel2'. As expected for the baseline susceptible variety Chardonnay, all but two strains (Pv2219_1 and Pv2221_1) displayed high sporulation, from 11.18 to 29.78% of leaf disc area covered with sporulation. On Seibel2, which carries the Rpv3.2 factor, all but one strain displayed sporulation area higher than 5.2% and nine displayed strong sporulation (*SpPr* > 14.0%). A single strain (Pv3116_1) did not infect Seibel2. This variety was also characterized by an absence of HR for most of the interactions.

The second group contained Regent, Solaris, Kunleany, 3160-12-3N, and RGM. For Regent, two phenotypes were distinguished. We found that 21 of the 33 strains studied displayed a low level of sporulation on Regent. Their sporulation area, from 0.38 to 6.76% and high levels of HR indicated effective resistance. The other 12 strains displayed much higher levels of sporulation (from 11.9 to 26.1% of the area displaying sporulation), with no induction
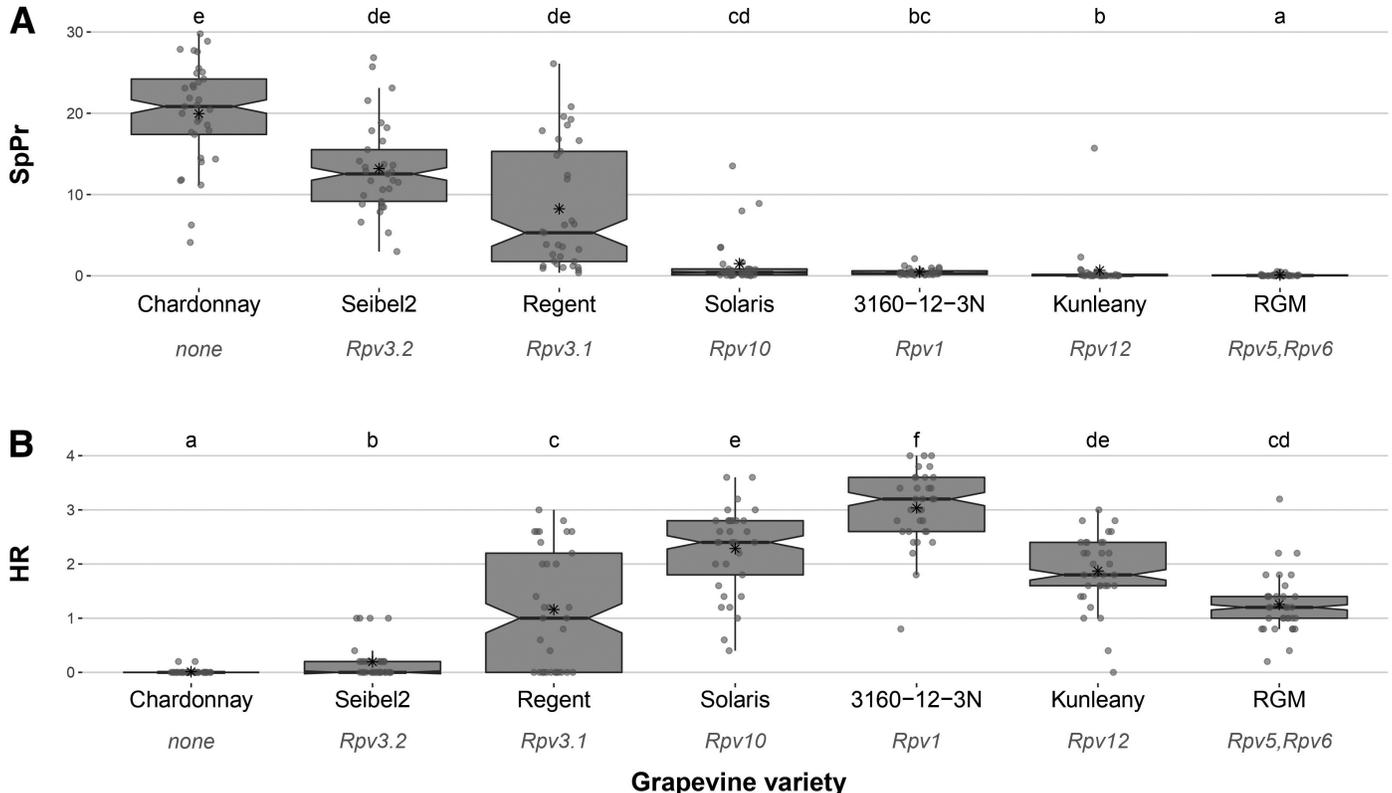
Fig. 3. Box plots of **A,** sporulation area (*SpPr*) and **B,** hypersensitive response (*HR*) intensity of the *Plasmopara viticola* strain set on the differential host range. Boxplots are based on 165 samples each (except for 3160-12-3N and Regent, with 164 samples). Horizontal lines correspond to the 25th, 50th, and 75th percentiles. Notches indicate the 95% confidence interval for comparing medians between boxplots, whereas vertical lines extend between the smallest and largest value no further than 1.5 × interquartile range (IQR). Dots indicate the sporulation average of the five replicates for each interaction. Black stars indicate the mean of the 165 samples. Letters indicate means significantly different between all pairwise inoculated hosts.

of HR. Solaris, Kunleany, 3160-12-3N and RGM, responded similarly to *P. viticola* infection: weak levels of sporulation (*SpPr* < 5.2%) and the induction of HR. However, a few interactions deviated from this general rule. On Solaris, three strains displayed intermediate levels of sporulation (Pv1356_1, 8.0%; Pv412_1, 8.9%; and Pv1419_1, 13.5%) while inducing HR for Pv412_1, and no HR for Pv1356_1 and Pv1419_1. It should be noted that Pv1356_1 and Pv1419_1, which were sampled from a variety carrying Rpv10, also sporulated on Rpv3. Furthermore, a single strain, Pv2543_1, was aggressive on Kunleany (*SpPr* = 15.7%) and induced no HR. This strain was sampled from a variety carrying the Rpv12 factor, which is also present in Kunleany. Finally, none of the *P. viticola* strains studied gave high levels of sporulation on 3160-12-3N or RGM, but most induced HR. RGM was characterized by low HR score, whereas high HR scores were obtained on 3160-12-3N. Besides the analysis at population level, the analysis of individual interactions reveals strong differences between strains which call for the definition of pathotypes based on the identification of resistance breakdowns.

Definition of resistance breakdown

We propose the classification of a strain as virulent against a given resistance factor if it (i) does not induce HR and (ii) has a high sporulation intensity. Thresholds are required to clarify this definition. In our experiment, a strain was considered virulent if (i) its mean HR intensity was strictly below 1 and (ii) its mean sporulation area was at least 50% that on Chardonnay.

This definition resulted in the classification of 28 out of 33 strains as virulent. These strains were involved in 37 cases of resistance breakdown among the 231 interactions involving a resistant variety (Fig. 4). Most of the resistance breakdowns detected concerned the Rpv3 haplotypes. We identified 23

strains as virulent on Seibel2 (all strains except Pv3116_1, Pv3003_1, Pv2664_1, Pv3069_1, Pv2868_1, Pv1356_1, Pv2578_1, Pv3199_11, Pv3191_1, Pv2534_1) and 12 as virulent on Regent (Pv1610_11, Pv2834_1, Pv413_11, Pv2547_1, Pv2546_1, Pv3003_1, Pv2664_, Pv3069_1, Pv2868_1, Pv1356_1, Pv412_11, and Pv1419_1). We identified one strain (Pv1419_1) that broke down the resistance mediated by Rpv10 and one (Pv2543_1) that broke down Rpv12 resistance (Fig. 5). Therefore, resistance breakdowns were identified for four of the six resistance factors tested (Rpv3.1, Rpv3.2, Rpv10, and Rpv12). In our set of 33 strains, 20 strains overcame a single resistance factor, 15 overcame only Rpv3.2, and five strains overcame only Rpv3.1. Virulence against two resistance factors was detected in seven strains and one strain broke down the resistance mediated by three resistance factors (Rpv3.1, Rpv3.2, and Rpv10) simultaneously. All multiple resistance breakdowns involved Rpv3.2.

We studied the correlation of sporulation and HR intensities between the inoculated varieties. For both sporulation and HR, we observed weak correlations between varieties (Supplementary Fig. S4A and B) which were always inferior to 0.5.

Identification of five *P. viticola* pathotypes

Based on the definition of virulence proposed above, we propose a nomenclature for pathotype definition. We developed a differential host panel composed of six varieties, each carrying one of the major resistance factors currently used in European breeding programs (2): '3160-12-3N' (Rpv1), 'Regent' (Rpv3.1), 'Seibel2' (Rpv3.2), 'Riparia Gloire de Montpellier' (Rpv5 and Rpv6), 'Solaris' (Rpv10), and 'Kunleany' (Rpv12). The differential hosts are listed in ascending order according to resistance factor numbering. Pathotypes are named according to the resistance factor they break down as follows: "vir" followed by the numbers of
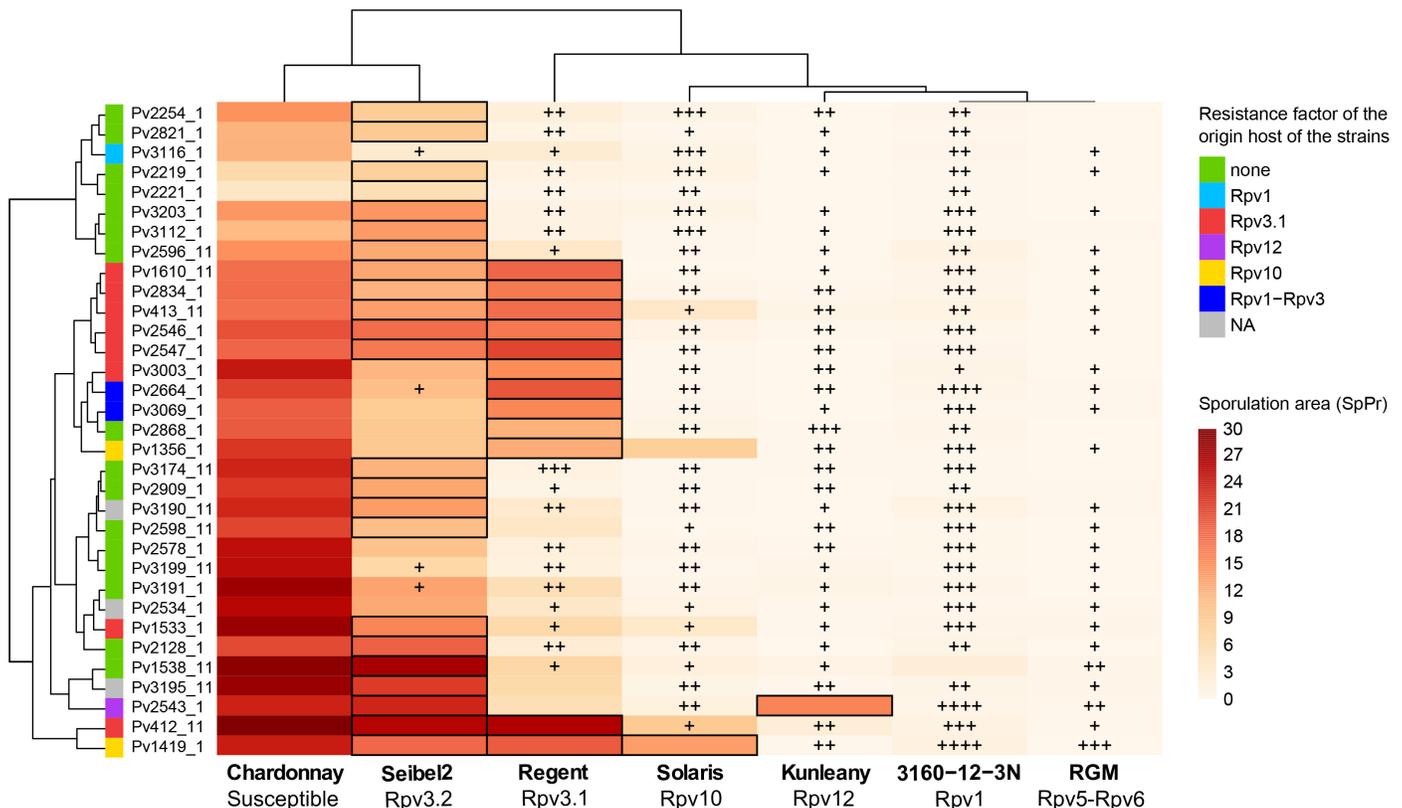
Fig. 4. Heatmap of the genotype−genotype interactions matrix for seven grapevine varieties and 33 downy mildew strains. The grapevine varieties are represented in columns and the strains in rows. Sporulation area is color-coded from light beige (0%, no sporulation) to deep red (30%, high levels of sporulation) based on the mean value for five replicates. The annotations in the heatmap cells indicate the mean hypersensitive response (HR) score: nothing for values between 0 and 1 (excluding 1); + for values between 1 and 2 (excluding 2); ++ for HR score between 2 and 3 (excluding 3); +++ for HR score between 3 and 4 (excluding 4); and ++++ for HR score equal to 4. The color annotations on the left side correspond to the resistance factor of the original host from which the strain was sampled. Dendrograms represent complete linkage clustering for the sporulation data. Framed cells indicate interactions with breakdown of resistance.

the resistance factors overcome, separated by commas. For example, pathotype vir3.1,10 overcomes the Rpv3.1 and Rpv10 factors. Strains unable to break down any of the resistance factors tested are labeled "avr".

The pathotypes identified and their geographic distribution are presented in Table 2 and Figure 6. The 33 strains tested included five avirulent strains that did not overcome any of the resistance factors. The remaining 28 strains each broke down at least one resistance factor, with 20 strains overcoming just one resistance factor each. The five strains that broke down Rpv3.1 resistance was annotated vir3.1 and the 15 strains breaking down the Rpv3.2 factor were annotated vir3.2. The six strains overcoming the two haplotypes Rpv3 (Rpv3.1 and Rpv3.2) were annotated vir3.1,3.2. We also detected a breakdown of the resistance mediated by the Rpv10 and Rpv12 factors. The strain breaking down the resistance mediated by Rpv10 and the two Rpv3 haplotypes was annotated vir3.1,3.2,10. Finally, the strain overcoming Rpv12 and Rpv3.2 was annotated vir3.2,12. Five pathotypes were observed: two pathotypes overcoming a single resistance factor (i.e., vir3.1 ($n = 5$) and vir3.2. ($n = 15$)), and three complex pathotypes overcoming two resistance factors (i.e., vir3.1,3.2 ($n = 6$) and vir3.2,12 ($n = 1$)) or three resistance factors

(i.e., vir3.1,3.2,10 ($n = 1$)). All of the virulent pathotypes involved Rpv3 (haplotype Rpv3.1 or Rpv3.2).

Discussion

The monitoring of virulence in plant pathogen populations requires the development of a reference protocol for the reliable and reproducible characterization of pathotypes. Reference protocols for pathotype definition have been established for several economically important plant pathogens (Spring et al. 2018), but no such tool is currently available for grapevine downy mildew. Furthermore, as all the known resistances to *P. viticola* are phenotypically quantitative (i.e., the strains manage to develop on resistant varieties, but to a lesser extent), the correct definition of resistance breakdown is not straightforward. Here, we present a first step towards a comprehensive method for characterizing the pathotypes of grapevine downy mildew strains.

The current reference method for measuring the leaf resistance of a grapevine variety to *P. viticola* is the OIV-452 descriptor (Anonymous 1983). This rating scale combines visual assessments of sporulation and HR in a single score (Fig. 1). Sporulation levels

Fig. 5. Breakdown of the resistance to downy mildew mediated by the Rpv10 and Rpv12 factors. The strain Pv1419_1 overcame the Rpv10 factor, as demonstrated by the absence of hypersensitive response (HR) and the high levels of sporulation on the Solaris leaf disc (57% of the sporulation observed on the susceptible variety), but not the Rpv12 factor, as demonstrated by the presence of HR and the absence of sporulation on the Kunleany leaf disc. Similarly, Pv2543_1 broke down the Rpv12 factor (67% of the sporulation observed on the susceptible variety) but not the Rpv10 factor. Symptoms were evaluated 6 days postinoculation on the abaxial side of the leaf disc.
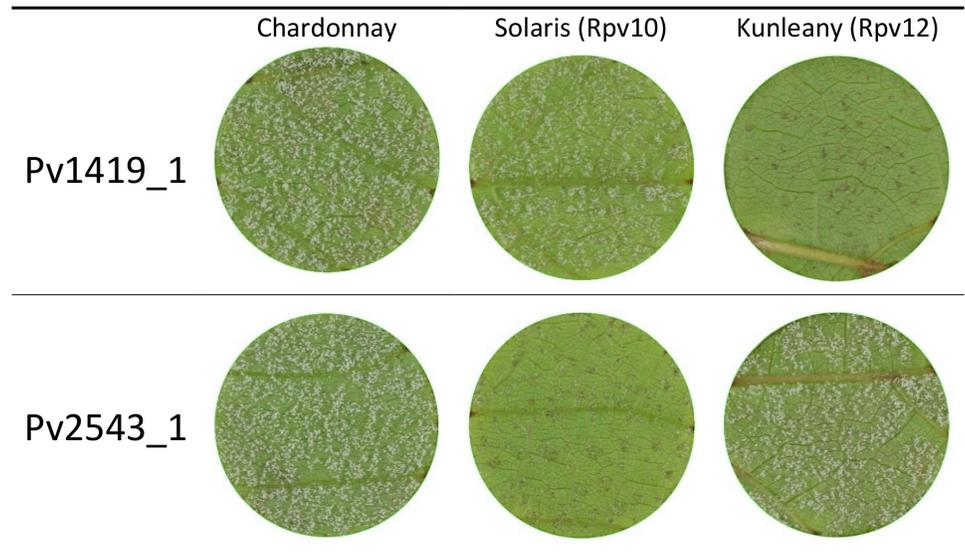
TABLE 2. Pathotype nomenclature for the 33 *Plasmopara viticola* strains[a]

Strains	Differential host range							Pathotype
	H1 Sensitive	H2 Rpv1	H3 Rpv3.1	H4 Rpv3.2	H5 Rpv5, Rpv6	H6 Rpv10	H7 Rpv12	
Pv3116–1; Pv2578–1; Pv3199–11; Pv3191–1; Pv2534–1	s	R	R	R	R	R	R	avr
Pv3003–1; Pv2664–1; Pv3069–1; Pv2868–1; Pv1356–1	s	R	s	R	R	R	R	vir3.1
Pv2254–1; Pv2821–1; Pv2219–1; Pv2221–1; Pv3203–1; Pv3112–1; Pv2596–11; Pv3174–11; Pv2909–1; Pv3190–11; Pv2598–1; Pv1533–1; Pv2128–1; Pv1538–11; Pv3195-11	s	R	R	s	R	R	R	vir3.2
Pv1610–11; Pv2834–1; Pv413–11; Pv2546–1; Pv2547–1; Pv412–11	s	R	s	s	R	R	R	vir3.1,3.2
Pv2543–1	s	R	R	s	R	R	s	vir3.2,12
Pv1419–1	s	R	s	s	R	s	R	vir3.1,3.2,10

[a] Differential hosts are shown in columns and *P. viticola* strains are shown in rows. s indicates that the host is susceptible, and R indicates that the host is resistant. List of hosts: H1 = Chardonnay; H2 = 3160-12-3N; H3 = Regent; H4 = Seibel2; H5 = Riparia Gloire de Montpellier, H6 = Solaris; and H7 = Kunleany.

provide an indication of the aggressiveness of *P. viticola*, whereas HR provides information about the strength of the resistance, a central feature of gene-for-gene interactions. The OIV-452 descriptor, initially designed for field evaluation, has been adapted for use in leaf discs assays conducted in laboratory conditions (Bellin et al. 2009): OIV-452-1. In addition to defining a rating score, the OIV-452-1 also recommends droplet inoculation. This method can have a major impact on sporulation and HR evaluations, and inoculation with droplets of sporangium suspension is probably the most widely used method for *P. viticola* (Boso and Kassemeyer 2008; Bove and Rossi 2020; Gómez-Zeledón et al. 2017; Li et al. 2016; Peressotti et al. 2010; Rumbolz et al. 2002; Schwander et al. 2012). It involves depositing a droplet of a standardized spore suspension in the center of the leaf disc. This method is simple to implement but flawed due to the characteristics of the leaf surface. In particular, leaf wettability affects the shape of the droplet and, therefore, the area of the leaf that is actually inoculated (Papierowska et al. 2019). By contrast, inoculation by spraying facilitates symptom scoring, as the whole leaf area is inoculated (Bellin et al. 2009; Blasi et al. 2011; Buonassisi et al. 2018; Calonnec et al. 2013; Zyprian et al. 2016). Spraying also makes it possible to calculate the proportion of the leaf disc area that is sporulating and the density of HR, facilitating comparisons between grapevine varieties.

In addition to the choice of inoculation method, the choice of scoring scale for symptoms also has an impact when the host range extends to resistant varieties. Sporulation and HR are weakly correlated in *P. viticola* ($r = 0.50$ in our dataset), especially when sporulation levels are low. Their combination into a single score, as in the OIV descriptor, requires a well-trained operator, to reduce the uncertainty of rating due to the human factor. We therefore recommend the separate assessment of sporulation and HR intensities. Sporulation is easy to score separately with the OIV-452-1 descriptor, and HR may be expressed as the number of punctate HR lesions per leaf disc (Fig. 1). Furthermore, the use of a particle counter or image analysis can provide a quantitative evaluation of sporu-

lation, with the additional advantage of being scorer-independent and easy to use. If both quantitative methods produce highly correlated measures, image analysis has the strong advantage to be a non-destructive method. This feature opens several experimental possibilities such as archiving part of a leaf disc in strains collections while extracting strain DNA on the remaining part to perform genomics studies. Moreover, image analysis enables to capture the dynamics of sporulation by taking pictures of the same discs in successive days. Thus, image analysis offers many crucial advantages over particle counter to pursue the study of the interaction between *P. viticola* genotypes and grapevine. It should be stressed that visual scoring is at least as good as quantitative methods for defining resistance breakdowns. Finally, our experimental design makes it possible to test the effect of the individual plants used as a source of leaf discs for inoculation (variable *IdP*), an effect highly significant for the intensities of both sporulation and HR. Even when grown in uniform greenhouse conditions, grapevine plants display considerable individual variability in terms of their susceptibility to downy mildew infections. Experimenters should therefore control for this effect by ensuring that replicates of a specific plant−pathogen interaction (leaf discs) each come from a different plant.

The definition of virulence proposed here takes into account the phenotypic traits measured at the individual level for the strain (difference between sporulation on susceptible and resistant plants). This choice could potentially lead to an overestimation of the virulence in populations. Indeed, when strains display lower levels of sporulation on the susceptible plant than the bulk of the pathogen population, there is a risk of their misclassification as virulent against a given resistance factor. This is the case for virulent strains Pv2219_1 and Pv2221_1 (vir3.2), which had a low sporulation area on Chardonnay (6.25 and 4.11%, respectively). As a means of avoiding this bias, the decision rule for defining virulence should incorporate comparisons with data obtained at population level, such as the mean level of sporulation on a given plant. This would
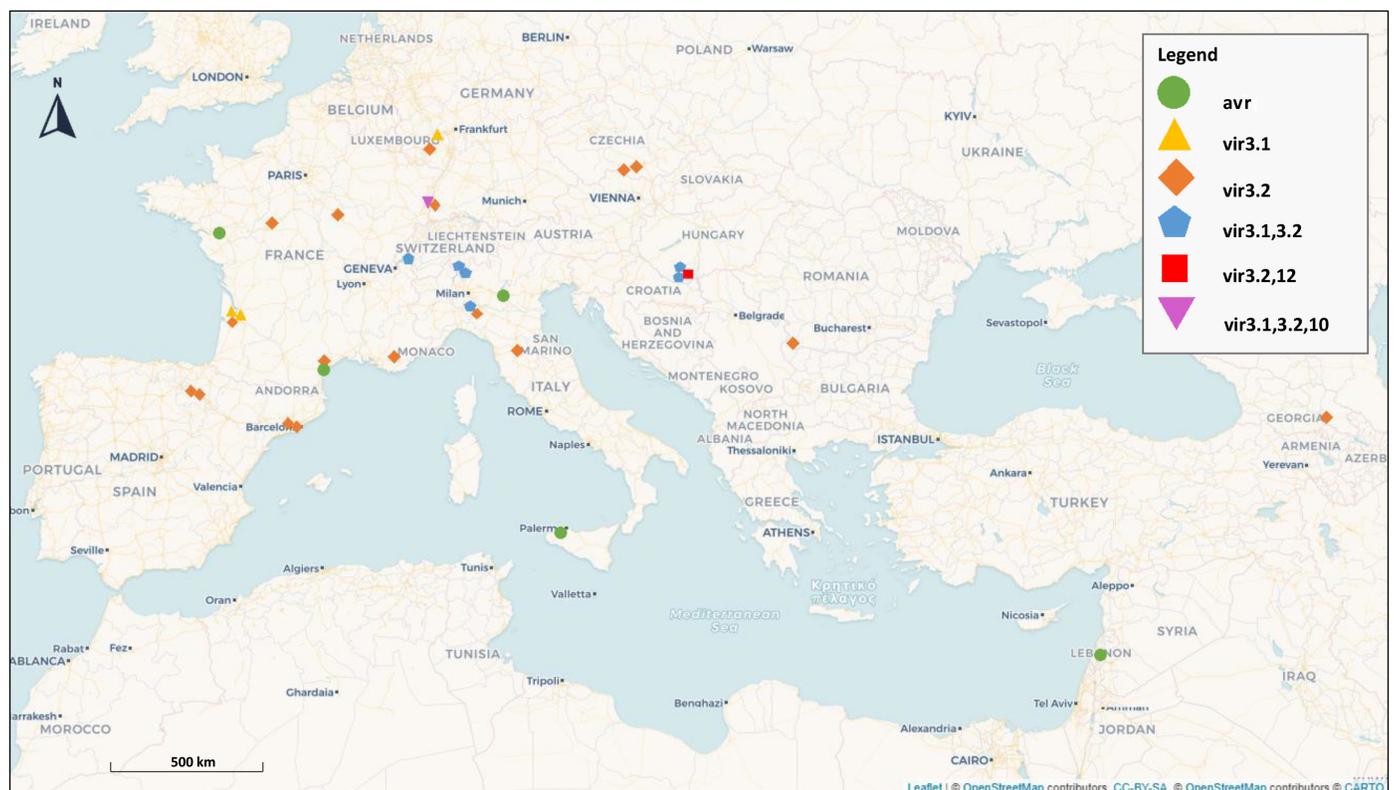
Fig. 6. Geographic distribution of pathotypes of the 33 *Plasmopara viticola* strains. The green circle represents avirulent strains (avr), the yellow triangles vir3.1, the orange diamonds vir3.2, the blue pentagons vir3.1,3.2, the red square vir3.2,12, and the violet inversed triangle vir3.1,3.2,10 pathotypes.

require the systematic phenotyping of at least 10 strains in pathotyping tests (most studies currently use only a few strains, as highlighted by Heyman et al. (2021) and Gómez-Zeledón et al. (2017), demonstrating the importance of sharing a collection of reference virulent and avirulent strains between research institutes.

The efficacy of any pathotyping methodology depends on the choice of a suitable differential host range and an appropriate nomenclature. The differential host range proposed here is composed of Chardonnay and six resistant varieties carrying the principal resistance factors currently used by European breeding institutes. The six resistant varieties retained for the differential host range each carry one major resistant factor in a complex genetic background. Monogenic lines are commonly used for the establishment of differential host ranges (Lebeda and Widrlechner 2003; Trojanová et al. 2017), but we decided to use grapevine varieties that are easily obtainable from vine nurseries or ampelographic collections. Many other downy mildew resistance factors have been identified in grapevine (Maul 2021) but have yet to be introgressed into *V. vinifera*. Finally, caution is required as regards the choice of Solaris, because this variety was recently reported to carry the Rpv3.3 resistance factor, in addition to Rpv10. The efficacy of Rpv3.3 for controlling *P. viticola* infection is largely unknown (Di Gaspero et al. 2012), but another representative variety carrying the Rpv10 factor (such as Muscaris, for example) should be preferred in the future for the development of the pathotyping method. For the naming of pathotypes, we propose a nomenclature listing the resistance factors overcome by the pathogen which provides immediate information about the R genes overcome by a strain. This classification, different from the one proposed by Cassagrande et al. (2011), is identical to the system currently used to describe the races of *Phytophthora infestans* (Mont.) de Bary (potato late blight) (Black et al. 1953; Fukue et al. 2018; Malcolmson 1969; Zhang and Kim 2007). It differs from numerical coding systems (triplet, quadruplet, sextet), which are currently used for species from the Peronosporaceae pathogenic to crops and closely related to *P. viticola*, such as *Plasmopara halstedii* (Farl.) Berl. & De Toni (the sunflower downy mildew agent), *Pseudoperonospora cubensis* (Berk. & M. A. Curtis) Rostovzev (the cucurbit downy mildew), and *Bremia lactucae* Regel (lettuce downy mildew) (Franco et al. 2020; Gulya et al. 1998; Lebeda and Widrlechner 2003). Numerical systems produce shorter pathotype names when the number of differential hosts is high, but they require an additional table to identify the resistance genes overcome (Black et al. 1953). The differential host range used to describe *P. viticola* virulence and the nomenclature system for pathotypes will obviously evolve with the deployment of new resistance factors. Furthermore, the methodology requires discussion and should be shared at continental, or international level, to ensure that it is truly useful to stakeholders. This would eventually lead to the sharing of the same range of plants (susceptible and resistant) between laboratories worldwide, together with a range of reference strains virulent against each of the resistance factors.

This study provides some of the first data concerning the pathotypes in European populations of downy mildew. We detected five pathotypes in Europe: two pathotypes with one virulence factor (vir3.1 and vir3.2), two pathotypes combining two virulences (vir3.1,3.2 and vir3.2,12), and one with three virulences (vir3.1,3.2,10). Our results indicate the absence of correlation between the varieties for sporulation and HR, indicating that Rpv1, Rpv3.1, Rpv3.2, Rpv5, Rpv6, Rpv10, and Rpv12 factors are based on different avr-genes−R-genes interactions. About 90% of the strains analyzed were virulent against at least one of the resistance factors currently available in grapevine. However, this figure probably largely overestimates the actual proportion of these strains in natural populations. Strains were not randomly sampled, with an overrepresentation of strains from vineyards planted with resistant varieties. Nevertheless, our results suggest that the Rpv3 factor (both Rpv3.1 and Rpv3.2) has now been largely overcome across Europe.

The breakdown of Rpv3 resistance was first described in 2010 on the Bianca variety in the Czech Republic (Peressotti et al. 2010). It was reported in Bordeaux vineyards (France), Pecs (Hungary), and the Rhine valley (France/Germany) 4 years later (Delmotte et al. 2014). Our results, and the findings recently published by Heyman et. al. (2021), confirm the ongoing adaptation of this pathogen to this resistance factor across European vineyards. However, it should be borne in mind that this resistance factor is present in many of the interspecific hybrids planted at the beginning of the 20th century. These hybrids were subsequently largely replaced by *V. vinifera* varieties from the 1950s onwards, but Rpv3 virulence alleles may have remained present at a very low frequency in *P. viticola* populations long after the elimination of the hybrids from vineyard landscapes. The current deployment of Rpv3-resistant varieties may therefore lead to the re-emergence of these alleles, accounting for the rapid response of populations to this resistance factor. This hypothesis could be tested by a molecular approach assessing the frequency of the mutation conferring virulence to the Rpv3 factor in *P. viticola* populations that have not been subjected to plant breeding pressure.

An important result of this study is the identification of the breakdown of grapevine resistance factors Rpv10 and Rpv12 in Europe. Indeed, we provide evidence for the first breakdown of Rpv10 resistance by a *P. viticola* isolate that was collected on Muscaris in Germany. Our results follow on from the findings of previous studies reporting an increase in the susceptibility of grapevine varieties carrying Rpv10 in Germany (Delmas et al. 2016; Gómez-Zeledón et al. 2017; Heyman et al. 2021). However, the resistance-breaking strain identified here (Pv1419_1) fully abolished the HR response of the plant, and such an abolition has not been observed before. Moreover, we report the discovery of a *P. viticola* strain (Pv2543_1) able to overcome the Rpv12 factor. This strain was collected in an experimental vineyard located in Pesc (Hungary) and planted with different grapevine genotypes carrying the Rpv12 factor. The Rpv12 factor was initially identified in the Asian grapevine species *V. amurensis* (Venuti et al. 2013). This breakdown of resistance follows on from the findings of both Li et al. (2015), who reported a *P. viticola* strain able to sporulate on resistant *V. amurensis* in China, and of Wingerter et al. (2021), who reported a strain from Switzerland that overcame both the Rpv3.1 and the Rpv12 factors. Altogether, these findings strongly suggest, therefore, that the deployment of the Rpv12 factor might lead to a shift in virulence of pathogen populations. The Rpv12 factor has been widely used in European breeding programs (Hungary, Italy, and Switzerland), leading to the creation of many different varieties, such as a recently released Sauvignac variety combining the Rpv3.1 and Rpv12 factors (Maul 2021). Further studies are required to assess the level of adaptation of *P. viticola* to Rpv12 and the threat this adaptation poses to the deployment of these new varieties.

The main strategy chosen by breeders to increase the durability of grapevine resistances is the pyramiding of resistance factors (Heyman et al. 2021; Schneider et al. 2019). The basic mechanism by which pyramids are likely to increase durability is that a pathogen must mutate simultaneously at several loci of its genome to overcome the defense mechanisms provided by the combination of resistance genes. If the mutations leading to adaptation are absent in the pathogen populations, the probability of this event is the product of the probabilities that the pathogen mutate at each individual locus (Mundt 2014). However, the durability of the pyramids can be compromised as soon as mutations are already present in the pathogen populations (Stam and McDonald 2018). This can typically be the case when the components of the pyramids have been already been deployed individually (Lof et al. 2017; Rimbaud et al. 2021). Our study shows that the deployment of monogenic varieties (Rpv3.1, Rpv3.2, Rpv10, and Rpv12), although currently limited, has already led to adaptation of *P. viticola* to these resistances. In this context, an important recommendation to favor the durability of pyramided grapevine varieties should be to limit as much as possible the deployments of these monogenic varieties and, if they are

to be deployed, to protect them with fungicide treatments in order to slow down the pathogen adaptation. However, even such a proactive strategy will not necessarily guarantee the sustainability of pyramids. Indeed, the mixed reproduction system of *P. viticola* combined to its large effective population size give it an overall strong evolutionary potential (McDonald and Linde 2002). Moreover, the resistance of grapevine to downy mildew being partial, avirulent or single virulent strains can multiply on pyramids and eventually mutate to acquire complementary virulences or compensatory mutations. Furthermore, the recombination of these strains at the end of the season could also lead to pyramid breakdowns. To this respect, we found that vir3.1 strains are already present on resistant varieties pyramiding the factors Rpv1 and Rpv3.1 (Pv2664_1 and Pv3069_1). This result highlights the importance to survey the dynamics of virulence emergence in the context of the deployment of pyramided varieties.

The method for characterizing grapevine downy mildew pathotypes described here provides a useful basis for the large-scale monitoring of this disease. We have shown that the proposed method satisfactorily highlights the known threat concerning the breakdown of the Rpv3 factor (both haplotypes Rpv3.1 and Rpv3.2), but it also highlighted the breakdown of the Rpv10 and Rpv12 factors. The sharing of this method internationally should make it possible to improve our understanding of the dynamics of adaptation in *P. viticola* and to provide information useful for the sustainable deployment of resistant varieties. Furthermore, the availability of a reliable pathotyping method paves the way for the identification of avirulence genes responsible of resistance breakdowns. Many genomic resources are available for *P. viticola* (Brilli et al. 2018; Dussert et al. 2019; Mestre et al. 2012; Yin et al. 2017), facilitating the investigation of regions of interest involved in resistance breakdown. Whole-genome association studies have recently successfully identified the *P. viticola* mating-type locus (Dussert et al. 2020), potentially making it possible to identify the genomic determinants responsible for the evolution of virulence in grapevine downy mildew.

Literature Cited

Anonymous. 1983. Pages 123-124 in: Descriptor list for grapevine varieties and *Vitis* species. 2nd ed. Office International de la Vigne et du Vin (OIV), Paris. www.vivc.de/docs/Code_descripteurs_2ed_EN.pdf

Bellin, D., Peressotti, E., Merdinoglu, D., Wiedemann-Merdinoglu, S., Adam-Blondon, A.-F., Cipriani, G., Morgante, M., Testolin, R., and Di Gaspero, G. 2009. Resistance to *Plasmopara viticola* in grapevine 'Bianca' is controlled by a major dominant gene causing localised necrosis at the infection site. Theor. Appl. Genet. 120:163-176.

Black, W., Mastenbroek, C., Mills, W. R., and Peterson, L. C. 1953. A proposal for an international nomenclature of races of *Phytophthora infestans* and of genes controlling immunity in *Solanum demissum* derivatives. Euphytica 2:173-179.

Blasi, P., Blanc, S., Wiedemann-Merdinoglu, S., Prado, E., Rühl, E. H., Mestre, P., and Merdinoglu, D. 2011. Construction of a reference linkage map of *Vitis amurensis* and genetic mapping of Rpv8, a locus conferring resistance to grapevine downy mildew. Theor. Appl. Genet. 123:43-53.

Blum, M., Waldner, M., and Gisi, U. 2010. A single point mutation in the novel PvCesA3 gene confers resistance to the carboxylic acid amide fungicide mandipropamid in *Plasmopara viticola*. Fungal Genet. Biol. 47:499-510.

Boso, S., and Kassemeyer, H. H. 2008. Different susceptibility of European grapevine cultivars for downy mildew. *Vitis*-J. Grapevine Res. 47:39-49.

Bove, F., and Rossi, V. 2020. Components of partial resistance to *Plasmopara viticola* enable complete phenotypic characterization of grapevine varieties. Sci. Rep. 10:1-12.

Brilli, M., Asquini, E., Moser, M., Bianchedi, P. L., Perazzolli, M., and Si-Ammour, A. 2018. A multi-omics study of the grapevine-downy mildew (*Plasmopara viticola*) pathosystem unveils a complex protein coding- and noncoding-based arms race during infection. Sci. Rep. 8:757.

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H. J., Machler, M., and Bolker, B. M. 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. R J. 9:378.

Buonassisi, D., Cappellin, L., Dolzani, C., Velasco, R., Peressotti, E., and Vezzulli, S. 2018. Development of a novel phenotyping method to assess downy mildew symptoms on grapevine inflorescences. Sci. Hortic. 236:79-89.

Calonnec, A., Wiedemann-Merdinoglu, S., Delière, L., Cartolaro, P., Schneider, C., and Delmotte, F. 2013. The reliability of leaf bioassays for predicting disease resistance on fruit: A case study on grapevine resistance to downy and powdery mildew. Plant Pathol. 62:533-544.

Casagrande, K., Falginella, L., Castellarin, S. D., Testolin, R., and Di Gaspero, G. 2011. Defense responses in Rpv3-dependent resistance to grapevine downy mildew. Planta 234:1097-1109.

Chen, W.-J., Delmotte, F., Cervera, S. R., Douence, L., Greif, C., and Corio-Costet, M.-F. 2007. At least two origins of fungicide resistance in grapevine downy mildew populations. Appl. Environ. Microbiol. 73:5162-5172.

Christensen, R. H. B. 2019. Ordinal—Regression Models for Ordinal Data. https://CRAN.R-project.org/package=ordinal

Delmas, C. E. L., Fabre, F., Jolivet, J., Mazet, I. D., Cervera, S. R., Delière, L., and Delmotte, F. 2016. Adaptation of a plant pathogen to partial host resistance: Selection for greater aggressiveness in grapevine downy mildew. Evol. Appl. 9:709-725.

Delmas, C. E. L., Mazet, I. D., Jolivet, J., Delière, L., and Delmotte, F. 2014. Simultaneous quantification of sporangia and zoospores in a biotrophic oomycete with an automatic particle analyzer: Disentangling dispersal and infection potentials. J. Microbiol. Methods 107:169-175.

Delmotte, F., Mestre, P., Schneider, C., Kassemeyer, H.-H., Kozma, P., Richart-Cervera, S., Rouxel, M., and Delière, L. 2014. Rapid and multiregional adaptation to host partial resistance in a plant pathogenic oomycete: Evidence from European populations of *Plasmopara viticola*, the causal agent of grapevine downy mildew. Infect. Genet. Evol. 27:500-508.

Di Gaspero, G., Copetti, D., Coleman, C., Castellarin, S. D., Eibach, R., Kozma, P., Lacombe, T., Gambetta, G., Zvyagin, A., Cindrić, P., Kovács, L., Morgante, M., and Testolin, R. 2012. Selective sweep at the Rpv3 locus during grapevine breeding for downy mildew resistance. Theor. Appl. Genet. 124:277-286.

Dussert, Y., Legrand, L., Mazet, I. D., Couture, C., Piron, M.-C., Serre, R.-F., Bouchez, O., Mestre, P., Toffolatti, S. L., Giraud, T., and Delmotte, F. 2020. Identification of the first oomycete mating-type locus sequence in the grapevine downy mildew pathogen, *Plasmopara viticola*. Curr. Biol. 30:3897-3907.e4.

Dussert, Y., Mazet, I. D., Couture, C., Gouzy, J., Piron, M.-C., Kuchly, C., Bouchez, O., Rispe, C., Mestre, P., and Delmotte, F. 2019. A high-quality grapevine downy mildew genome assembly reveals rapidly evolving and lineage-specific putative host adaptation genes. Genome Biol. Evol. 11:954-969.

Fischer, B. M., Salakhutdinov, I., Akkurt, M., Eibach, R., Edwards, K. J., Töpfer, R., and Zyprian, E. M. 2004. Quantitative trait locus analysis of fungal disease resistance factors on a molecular map of grapevine. Theor. Appl. Genet. 108:501-515.

Fontaine, M. C., Austerlitz, F., Giraud, T., Labbé, F., Papura, D., Richard-Cervera, S., and Delmotte, F. 2013. Genetic signature of a range expansion and leap-frog event after the recent invasion of Europe by the grapevine downy mildew pathogen *Plasmopara viticola*. Mol. Ecol. 22:2771-2786.

Fontaine, M. C., Labbé, F., Dussert, Y., Delière, L., Richard-Cervera, S., Giraud, T., and Delmotte, F. 2021. Europe as a bridgehead in the worldwide invasion history of grapevine downy mildew, *Plasmopara viticola*. Curr. Biol. 31:2155-2166.

Foria, S., Copetti, D., Eisenmann, B., Magris, G., Vidotto, M., Scalabrin, S., Testolin, R., Cipriani, G., Wiedemann-Merdinoglu, S., Bogs, J., Gaspero, G. D., and Morgante, M. 2020. Gene duplication and transposition of mobile elements drive evolution of the Rpv3 resistance locus in grapevine. Plant J. 101:529-542.

Franco, C. A., Marin, M. V., Silva, E. H. C., Soares, R. S., Candido, W. S., Souza, L. N., Caprio, C. H., Vidal, R. L., Panizzi, R. C., and Braz, L. T. 2020. Monitoring virulence of *Bremia lactucae* as a breeding tool against lettuce downy mildew from south and southwest Brazilian regions. Eur. J. Plant Pathol. 159:179-189.

Fukue, Y., Akino, S., Osawa, H., and Kondo, N. 2018. Races of *Phytophthora infestans* isolated from potato in Hokkaido, Japan. J. Gen. Plant Pathol. 84:276-278.

Gessler, C., Pertot, I., and Perazzolli, M. 2011. *Plasmopara viticola*: A review of knowledge on downy mildew of grapevine and effective disease management. Phytopathol. Mediterr. 50:3-44.

Gómez-Zeledón, J., Kaiser, M., and 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.

Gómez-Zeledón, J., Kaiser, M., and Spring, O. 2017. Exploring host−pathogen combinations for compatible and incompatible reactions in grapevine downy mildew. Eur. J. Plant Pathol. 149:1-10.

Gómez-Zeledón, J., Zipper, R., and Spring, O. 2013. Assessment of phenotypic diversity of *Plasmopara viticola* on *Vitis* genotypes with different resistance. Crop Prot. 54:221-228.

Gulya, T. J., Tourvieille de Labrouche, D., Masirevic, S., Penaud, A., Rashid, K. Y., and Viranyi, F. 1998. Proposal for standardized nomenclature and identification of races of *Plasmopara halstedii* (sunflower downy mildew). Pages 130-136 in: Proc. Sunflower Downy Mildew Symposium.

Hartig, F., and Lohse, L. 2020. DHARMa: Residual Diagnostics for Hierarchical (Multi-Level/Mixed) Regression Models. https://CRAN.R-project.org/package=DHARMa

Heyman, L., Höfle, R., Kicherer, A., Trapp, O., Ait Barka, E., Töpfer, R., and Höfte, M. 2021. The durability of quantitative host resistance and variability in pathogen virulence in the interaction between European grapevine cultivars and *Plasmopara viticola*. Front. Agron. 3:39.

Johnson, R., Stubbs, R. W., Fuchs, E., and Chamberlain, N. H. 1972. Nomenclature for physiologic races of *Puccinia striiformis* infecting wheat. Trans. Br. Mycol. Soc. 58:475-480.

Kamoun, S., Furzer, O., Jones, J. D. G., Judelson, H. S., Ali, G. S., Dalio, R. J. D., Roy, S. G., Schena, L., Zambounis, A., Panabières, F., Cahill, D., Ruocco, M., Figueiredo, A., Chen, X.-R., Hulvey, J., Stam, R., Lamour, K., Gijzen, M., Tyler, B. M., Grünwald, N. J., Mukhtar, M. S., Tomé, D. F. A., Tör, M., Van Den Ackerveken, G., McDowell, J., Daayf, F., Fry, W. E., Lindqvist-Kreuze, H., Meijer, H. J. G., Petre, B., Ristaino, J., Yoshida, K., Birch, P. R. J., and Govers, F. 2015. The top 10 oomycete pathogens in molecular plant pathology. Mol. Plant Pathol. 16:413-434.

Kolde, R. 2019. pheatmap: Pretty Heatmaps. https://CRAN.R-project.org/package=pheatmap

Lebeda, A., and Widrlechner, M. P. 2003. A set of Cucurbitaceae taxa for differentiation of *Pseudoperonospora cubensis* pathotypes. Z. Pflanzenkh. Pflanzenschutz/J. Plant Dis. Prot. 110:337-349.

Lenth, R. V. 2016. Least-squares means: The R package lsmeans. J. Stat. Softw. 69:1-33.

Li, X., Wu, J., Yin, L., Zhang, Y., Qu, J., and Lu, J. 2015. Comparative transcriptome analysis reveals defense-related genes and pathways against downy mildew in *Vitis amurensis* grapevine. Plant Physiol. Biochem. 95:1-14.

Li, X., Yin, L., Ma, L., Zhang, Y., An, Y., and Lu, J. 2016. Pathogenicity variation and population genetic structure of *Plasmopara viticola* in China. J. Phytopathol. 164:863-873.

Lof, M. E., de Vallavieille-Pope, C., and van der Werf, W. 2017. Achieving durable resistance against plant diseases: Scenario analyses with a national-scale spatially explicit model for a wind-dispersed plant pathogen. Phytopathology 107:580-589.

Malcolmson, J. F. 1969. Races of *Phytophthora infestans* occurring in Great Britain. Trans. Br. Mycol. Soc. 53:417-IN2.

Marguerit, E., Boury, C., Manicki, A., Donnart, M., Butterlin, G., Némorin, A., Wiedemann-Merdinoglu, S., Merdinoglu, D., Ollat, N., and Decroocq, S. 2009. Genetic dissection of sex determinism, inflorescence morphology and downy mildew resistance in grapevine. Theor. Appl. Genet. 118:1261-1278.

Maul, E. 2021. *Vitis* International Variety Catalogue. Julius Kühn-Institut. https://www.vivc.de/

McDonald, B. A., and Linde, C. 2002. The population genetics of plant pathogens and breeding strategies for durable resistance. Euphytica 124:163-180.

Merdinoglu, D., Schneider, C., Prado, E., Wiedemann-Merdinoglu, S., and Mestre, P. 2018. Breeding for durable resistance to downy and powdery mildew in grapevine. OENO One 52:203-209.

Merdinoglu, D., Wiedeman-Merdinoglu, S., Coste, P., Dumas, V., Haetty, S., Butterlin, G., and Greif, C. 2003. Genetic analysis of downy mildew resistance derived from *Muscadinia rotundifolia*. Acta Hortic. 603:451-456.

Mestre, P., Piron, M.-C., and Merdinoglu, D. 2012. Identification of effector genes from the phytopathogenic oomycete *Plasmopara viticola* through the analysis of gene expression in germinated zoospores. Fungal Biol. 116:825-835.

Millardet, A. 1881. Notes Sur Les Vignes Américaines et Opuscules Divers Sur Le Même Sujet. Nabu Press, Bordeaux.

Moroldo, M., Paillard, S., Marconi, R., Fabrice, L., Canaguier, A., Cruaud, C., De Berardinis, V., Guichard, C., Brunaud, V., Le Clainche, I., Scalabrin, S., Testolin, R., Di Gaspero, G., Morgante, M., and Adam-Blondon, A.-F. 2008. A physical map of the heterozygous grapevine 'Cabernet Sauvignon' allows mapping candidate genes for disease resistance. BMC Plant Biol. 8:66.

Mundt, C. C. 2014. Durable resistance: A key to sustainable management of pathogens and pests. Infect. Genet. Evol. 27:446-455.

Papierowska, E., Mazur, R., Stańczyk, T., Beczek, M., Szewińska, J., Sochan, A., Ryżak, M., Szatyłowicz, J., and Bieganowski, A. 2019. Influence of leaf surface wettability on the drop splash phenomenon. Agric. For. Meteorol. 279:107762.

Peressotti, E., Wiedemann-Merdinoglu, S., Delmotte, F., Bellin, D., Di Gaspero, G., Testolin, R., Merdinoglu, D., and Mestre, P. 2010. Breakdown of resistance to grapevine downy mildew upon limited deployment of a resistant variety. BMC Plant Biol. 10:147.

R Core Team. 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.r-project.org/

Rimbaud, L., Fabre, F., Papaïx, J., Moury, B., Lannou, C., Barrett, L. G., and Thrall, P. H. 2021. Models of plant resistance deployment. Annu. Rev. Phytopathol. 59:125-152.

Rouxel, M., Mestre, P., Baudoin, A., Carisse, O., Delière, L., Ellis, M. A., Gadoury, D., Lu, J., Nita, M., Richard-Cervera, S., Schilder, A., Wise, A., and Delmotte, F. 2014. Geographic distribution of cryptic species of *Plasmopara viticola* causing downy mildew on wild and cultivated grape in eastern North America. Phytopathology 104:692-701.

Rouxel, M., Mestre, P., Comont, G., Lehman, B. L., Schilder, A., and Delmotte, F. 2013. Phylogenetic and experimental evidence for host-specialized cryptic species in a biotrophic oomycete. New Phytol. 197:251-263.

Rumbolz, J., Wirtz, S., Kassemeyer, H.-H., Guggenheim, R., Schäfer, E., and Büche, C. 2002. Sporulation of *Plasmopara viticola*: Differentiation and light regulation. Plant Biol. 4:413-422.

Schneider, C., Onimus, C., Prado, E., Dumas, V., Wiedemann-Merdinoglu, S., Dorne, M., Lacombe, M., Piron, M., Umar-Faruk, A., Duchêne, E., Mestre, P., and Merdinoglu, D. 2019. INRA-ResDur: The French grapevine breeding programme for durable resistance to downy and powdery mildew. Acta Hortic. 1248:207-214.

Schwander, F., Eibach, R., Fechter, I., Hausmann, L., Zyprian, E., and Töpfer, R. 2012. Rpv10: A new locus from the Asian *Vitis* gene pool for pyramiding downy mildew resistance loci in grapevine. Theor. Appl. Genet. 124:163-176.

Spring, O., Gomez-Zeledon, J., Hadziabdic, D., Trigiano, R. N., Thines, M., and Lebeda, A. 2018. Biological characteristics and assessment of virulence diversity in pathosystems of economically important biotrophic oomycetes. Crit. Rev. Plant Sci. 37:439-495.

Stam, R., and McDonald, B. A. 2018. When resistance gene pyramids are not durable—the role of pathogen diversity. Mol. Plant Pathol. 19:521-524.

Trojanová, Z., Sedlářová, M., Gulya, T. J., and Lebeda, A. 2017. Methodology of virulence screening and race characterization of *Plasmopara halstedii*, and resistance evaluation in sunflower–A review. Plant Pathol. 66:171-185.

Van Ettekoven, K., and Van der Arend, A. 1999. Identification and denomination of "new" races of *Bremia lactucae*. Pages 171-175 in: EUCARPIA leafy vegetables 1999, Proceedings of the EUCARPIA meeting on leafy vegetables genetics and breeding. A. Lebeda and E. Kristková, eds. EUCARPIA, Germany.

Venuti, S., Copetti, D., Foria, S., Falginella, L., Hoffmann, S., Bellin, D., Cindrić, P., Kozma, P., Scalabrin, S., Morgante, M., Testolin, R., and Gaspero, G. D. 2013. Historical introgression of the downy mildew resistance gene rpv12 from the Asian species *Vitis amurensis* into grapevine varieties. PLoS One 8:e61228.

Wei, T. S. 2021. R package 'corrplot': Visualization of a Correlation Matrix. https://cran.r-project.org/web/packages/corrplot/corrplot.pdf

Welter, L. J., Göktürk-Baydar, N., Akkurt, M., Maul, E., Eibach, R., Töpfer, R., and Zyprian, E. M. 2007. Genetic mapping and localization of quantitative trait loci affecting fungal disease resistance and leaf morphology in grapevine (*Vitis vinifera* L.). Mol. Breed. 20:359-374.

Wingerter, C., Eisenmann, B., Weber, P., Dry, I., and Bogs, J. 2021. Grapevine Rpv3-, Rpv10-, and Rpv12-mediated defense responses against *Plasmopara viticola* and the impact of their deployment on fungicide use in viticulture. BMC Plant Biol. 21:470.

Yin, L., An, Y., Qu, J., Li, X., Zhang, Y., Dry, I., Wu, H., and Lu, J. 2017. Genome sequence of *Plasmopara viticola* and insight into the pathogenic mechanism. Sci. Rep. 7:46553.

Zhang, X.-Z. N. A. U., and Kim, B. S. K. N. U. 2007. Physiological races of *Phytophthora infestans* in Korea. Plant Pathol. J. 23:219-222.

Zyprian, E., Ochßner, I., Schwander, F., Šimon, S., Hausmann, L., Bonow-Rex, M., Moreno-Sanz, P., Grando, M. S., Wiedemann-Merdinoglu, S., Merdinoglu, D., Eibach, R., and Töpfer, R. 2016. Quantitative trait loci affecting pathogen resistance and ripening of grapevines. Mol. Genet. Genom. 291:1573-1594.