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The reliability of leaf bioassays for predicting disease resistance on fruit A case study on grapevine resistance to downy and powdery mildew

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This study was designed to assess the reliability of grapevine leaf bioassays for predicting disease resistance on fruit in the field. The efficacy of various grapevine quantitative trait loci (QTLs) for conferring resistance to downy and powdery mildew was evaluated in bioassays and in a 2-year field experiment for downy mildew. The resistance genes studied were inherited from *Muscadinia rotundifolia* (*Rpv1* and *Run1*) and from American *Vitis* species through cv. Regent (QTLRgP and QTLRgD). In bioassays, genotypes carrying *Run1* blocked powdery mildew development at early stages. Genotypes combining *Run1* with QTLRgP displayed no greater level of resistance. For downy mildew, genotypes carrying *Rpv1* and/or QTLRgD were more resistant than the susceptible cv. Merlot, and showed a high level of leaf resistance in the field (<10% severity). Disease levels on bunches were much higher than those on leaves, with a high variability between *Rpv1* genotypes (1–48%). A Bayesian decision theory framework predicted that an OIV-452 threshold of 5 in leaf bioassays allowed accurate selection of grapevine genotypes (P = 0·83) with satisfactory disease severity on bunches. Therefore, this study validates that the use of early bioassays on leaves, as currently performed by grapevine breeders, ensures a satisfactory level of resistance to downy mildew of bunches in the field.

Keywords: *Erysiphe necator*, marker-assisted selection, perennial plant, *Plasmopara viticola*, receiver operating characteristic

Introduction

The breeding of perennial crops is a complex, long-term process, extending over several decades. The main reasons for this are the long lifespans of the crops and the difficulty of obtaining fertile progeny from crops more easily multiplied by vegetative propagation. Moreover, the traits of interest in crops such as grapevine (wood quality, fruit quality, disease resistance, etc.) can often be observed and screened only at late stages of development and require assessment over a number of years at different locations. Systems for accelerating the selection process are therefore required. During recent decades, developments in molecular biology and genetics have allowed the advent of DNA marker-assisted selection (MAS) (Lande & Thompson, 1990). MAS can indeed greatly enhance the identification of sources of variation and selection of complex traits that are phenotypically difficult or time-consuming to evaluate. Disease resistance is typically a quantitative trait for which MAS procedures can be efficiently developed. In perennial crops, the use of MAS can greatly decrease the time taken to select for resistance and make it possible to rapidly identify genotypes combining several disease resistance genes (Collard & Mackill, 2008).

Screening for markers correlated with disease resistance is dependent on the use of efficient and reliable pathogenicity tests. Laboratory tests on leaves from seedlings are easy to perform on a large number of genotypes, with controlled sources of inoculum. However, the use of such tests at an early stage of development may not necessarily provide a reliable indication of subsequent disease resistance on plants at later stages of development or in different organs. In fact, it remains unclear whether plant genotypes selected on the basis of the resistance of their leaves in the laboratory at an early stage are likely to display high levels of resistance on fruit, either directly or indirectly (as a result of reduced inoculum coming from leaves). Indeed, a strong relationship between disease resistance in leaves and fruit has been reported for some host/pathogen systems, e.g. durian/*Phytophthora* (Vawdrey *et al.*, 2005), cocoa/*Phytophthora* diseases (Iwaro *et al.*, 1997), whereas a weaker relationship has been reported for other systems: blueberry/anthracnose

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(*Colletotrichum acutatum*) (Ehlenfeldt *et al.*, 2006) and apple/scab (*Venturia inaequalis*) (Liebhard *et al.*, 2003). In some systems with a weak genotype × environment interaction (e.g. cocoa/*Phytophthora*) the use of MAS has already been integrated into systems for selecting resistance. In pathosystems in which the expression of resistance depends strongly on the effects of organ age or, more generally, on the environment (apple/powdery mildew, peach/powdery mildew, coffee/berry disease, grapevine/powdery and downy mildew), the routine use of MAS (Foulongne *et al.*, 2003) would require a more detailed understanding of the stability of quantitative trait loci (QTLs) for disease resistance as a function of genetic background and environment, at various phenological stages and in different organs (Welter *et al.*, 2007).

Receiver operating characteristic (ROC) curves and Bayes' theorem could be valuable predictive tools in breeding programmes, especially in determining the probability of selecting plant genotypes with high levels of resistance on leaves and on fruit in the field based on early laboratory bioassays. ROC curves have proven useful for the evaluation of diagnostic tests in clinical pathology (Metz, 1978) and have more recently been applied to plant pathology to validate the performance of a model in disease prediction (Yuen *et al.*, 2002; Madden *et al.*, 2007).

Cultivated vine (*Vitis vinifera*) is a good example of a perennial crop in which selection for disease resistance is highly challenging. *Vitis vinifera* originated from Eurasia and has been domesticated and cultivated for about 5000 years. Unfortunately, *V. vinifera* carries no genes conferring resistance to its most damaging pathogens, *Erysiphe necator* (causal agent of powdery mildew) and *Plasmopara viticola* (causal agent of downy mildew), both of which originate from North America. However, several *Vitis* species that co-evolved with these pathogens and display moderate to high levels of resistance are present in North America (Gee *et al.*, 2008). Thus, wild American *Vitis* spp. have been widely used as powdery and downy mildew-resistant parents in interspecific crosses. However, these classical crossbreeding approaches are confronted with strong inbreeding depression of progenies, the possible difference in chromosome number between species and the tendency of wild species to be dioecious. In this context, MAS is a promising tool to identify QTLs for leaf and bunch resistance to *E. necator* and *P. viticola* (Fischer *et al.*, 2004; Akkurt *et al.*, 2007).

This study was carried out in the advanced stages of a breeding programme aiming to select genotypes most likely to confer durable resistance (according to the definition of Johnson (1984) a resistance that remains effective during its prolonged and widespread use in an environment favourable to the disease) to both powdery and downy mildew, together with favourable oenological traits.

The population studied was segregating for various resistance genes conferring strong or moderate levels of resistance and had a mixed genetic background: QTLs for resistance to powdery and downy mildews originating from *Muscadinia rotundifolia* (Pauquet *et al.*, 2001; Barker *et al.*, 2005) and from cv. Regent (Fischer *et al.*, 2004; Akkurt *et al.*, 2007; Eibach *et al.*, 2007). This population showed enough variation for phenotypic traits to be used as a case study to address the following questions: (i) what is the relationship between leaf resistance in bioassays and resistance in the field? (ii) how effective and how variable is the resistance conferred by the various QTLs, alone or in combination, on leaves and bunches? and (iii) how effective are bioassays on leaves to predict bunch resistance to downy mildew in the field? These questions were addressed by evaluating the level of resistance of this population to both *E. necator* and *P. viticola* in bioassays, in the field, and on different organs of the plant (leaves and bunches). A Bayesian decision theory framework (ROC curve) was applied to evaluate the performance of a leaf bioassay in selection for genotypes resistant to disease in the field.

Materials and methods

Plant material and field experiment design

The pseudo-F_1 progeny studied resulted from a cross between 3082-1-42 and Regent, realized within the framework of an INRA (France) – JKI (Germany) collaboration. 3082-1-42 is a genotype derived from a cross between *M. rotundifolia* and *V. vinifera* followed by four backcrosses with *V. vinifera* cv. Regent, an offspring of Chambourcin (12·417 SV × 7053 Seibel) and Diana (Sylvaner × Müller Thurgau). Regent was registered in 1995 in the German catalogue of grapevine varieties and currently over 2% of German vineyards are planted with this cultivar (2100 ha in 2010). The parent 3082-1-42 transmitted *Run1* (total resistance to powdery mildew) and *Rpv1* (partial resistance to downy mildew) and the parent Regent transmitted one QTL of resistance to powdery mildew (called QTLRgP in this paper) and one QTL for partial resistance to downy mildew (called QTLRgD in this paper) (Fischer *et al.*, 2004; Akkurt *et al.*, 2007; Eibach *et al.*, 2007). *Run1* was first introduced into the *V. vinifera* genome using pseudo-backcross strategies between *V. vinifera* cultivars and a hybrid arising from a cross between *M. rotundifolia* and *V. vinifera* cv. Malaga (Bouquet, 1986), and the markers linked to the powdery mildew resistance were first identified in the different backcross segregating populations (Pauquet *et al.*, 2001). Later studies with the same populations identified a second resistance locus, *Rpv1*, bringing resistance to downy mildew (Merdinoglu *et al.*, 2003). *Run1* and *Rpv1* were found co-segregating in the population under study. QTLRgP and QTLRgD were first identified in a cross between cvs Regent and Lemberger (Fischer *et al.*, 2004).

In total, 36 genotypes from the segregating population, with different levels of resistance to powdery and downy mildew, were planted in Bordeaux in 2004.

The experimental design consisted of four consecutive vines for each of the 36 genotypes, the two parents and a susceptible control variety (Merlot) planted in a randomized design. Plants were left untreated for the 2 years of the study.

Genetic characterization of grape genotypes

For each grape genotype, in September 2007, young expanding leaves were collected and 80 mg ground to a fine powder in liquid nitrogen. Total DNA was extracted with the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions.

Screening for *Rpv1* (and *Run1* genes) was carried out as described by Merdinoglu *et al.* (2003) using two linked SSR markers: VMC_8g9 and VMC_4f3. QTLs for resistance inherited from cv. Regent were screened with two SCAR markers: ScORA7-760 was used to screen for the QTL for leaf resistance to powdery mildew (Akkurt *et al.*, 2007) and ScPRA14-464 was used to screen for the downy mildew QTL (E. Zyprian, Julius Kühn Institute, Germany, personal communication). PCR for SSR loci was carried out in a standard reaction volume of 15 μL. Primer pairs were labelled with ABI fluorescent dyes and analysed by capillary electrophoresis. PCR for SCAR markers was carried out in standard reaction volumes of 25 μL and PCR products were separated by electrophoresis in a 1·5% agarose gel.

For each disease, genotypes were classified into one of the four possible gene combinations: for powdery mildew resistance, [Run1 QTLRgP] refers to genotypes with the *Run1* gene and the powdery mildew QTL from Regent, [Run1 -] refers to genotypes with *Run1* only, [- QTLRgP] refers to genotypes with the powdery mildew QTL from Regent and [- -] refers to genotypes with none of the resistance genes. Similarly, the four combinations for downy mildew resistance were as follows: [Rpv1 QTLRgD], [Rpv1 -], [- QTLRgD] and [- -].

Bioassays

Powdery and downy mildew isolates

A single-spored isolate of *E. necator* (S7, group B) was obtained from a *V. vinifera* cv. Cabernet Sauvignon plant in La Grande Ferrade (Bordeaux, France) in 2007. The isolate was multiplied on Cabernet Sauvignon leaves following the method described by Cartolaro & Steva (1990). For downy mildew, the *P. viticola* isolate was collected in 2002 from *V. vinifera* cv. Gewürztraminer in Bergheim (France). Propagation of this isolate was conducted by infecting detached leaves from seedlings of *V. vinifera* cv. Muscat Ottonel cultivated on stone wool as described in Peressotti *et al.* (2010).

Powdery mildew bioassay

Healthy leaves were harvested in the field. On 25 April 2007 (average of seven leaves/shoot), one young leaf per vine (one or two leaves below the last expanded leaf) was sampled for each genotype (four leaves = four repetitions/genotype = one leaf per vine), for the susceptible control variety (Merlot) and for both parents: Regent and 3082-1-42. Leaves were disinfected by incubation for 10 min in 50 g L^{-1} sodium hypochlorite, and a 16-mm sample disc was taken from each leaf and placed on medium containing 20 g L^{-1} agar in a Petri dish, adaxial surface uppermost. Five host genotypes were tested per Petri dish. Each Petri dish included one positive control for infection based on leaf discs derived from cuttings of Cabernet Sauvignon. Each of the 36 genotypes plus the controls (Merlot and parents from the field) were inoculated in one settling tower (four towers for infection of the four repetitions/genotype) by blowing 600–800 spores cm^{-2} of isolate S7 onto the leaf discs. Thirteen days after inoculation, all discs were observed under a stereomicroscope (×40) to assess if the mycelium was present especially for non-sporulating samples. Sporulation was assessed by a Coulter cell counter (Multisizer III) after shaking each disc in 20 mL isotonic solution containing one drop of a non-ionic dispersant (Nacconol *90F*); particles between 17 and 37 μm were counted in samples of 500 μL solution. For all genotypes showing no visual sporulation an adhesive tape test with cotton blue staining procedure was performed (Cartolaro & Steva, 1990) and samples were observed under the microscope.

Downy mildew bioassay

Leaves were detached from wood cuttings harvested in two replicates in a greenhouse at 25°C. The fourth and fifth leaves from the apex were detached and rinsed with distilled water. Two plant replicates for each genotype were used and for each leaf 12 discs of 1-cm diameter were excised with a cork borer on a PVC pad. Leaf discs from the two leaves were bulked and distributed over two Petri dishes with the abaxial surface up, obtaining 22 leaf discs for each replicate. The bottom of each dish was covered in advance with filter paper dampened with 4 mL sterile distilled water. Discs were inoculated by spraying with a sporangial suspension at 10^5 sporangia mL^{-1}. Petri dishes were incubated at 21°C for 7 days. All 36 genotypes were tested. Resistance was scored on a visual scale (five levels) similar to that of OIV-452 for leaves (IPGRI, 1997), taking into account sporulation intensity and necroses (the higher the score, the greater the degree of resistance: 1 = very low, 3 = low, 5 = medium, 7 = high, 9 = very high or total). For each replicate one average OIV-452 score was given. The score for each genotype was the average of the two replicates. Sporulation assessed with a Coulter counter on 12 discs from one replicate showed a good correlation (Spearman's R^2 = 0·82) between the OIV-452 score and sporulation.

Field assessments

For downy mildew, disease was assessed in the vineyard (cf. experimental design) on 30 June 2007 and 7 July 2008 (before and at bunch closure, respectively). In 2007,

the susceptibility of each vine to downy mildew was determined by a visual assessment of severity on leaves (global assessment per vine of the proportion of leaf area with downy mildew symptoms). In 2008, a more precise assessment was performed on a sample of 30 leaves per vine (randomly chosen on the whole canopy), with the calculation of average severity (percentage of leaf area with downy mildew symptoms). In both years, disease severity was scored for each bunch of each vine (percentage of area diseased, corresponding to sporulating or discoloured berries). At the start of September, all bunches were observed for each vine and classified as 'harvestable' or 'non-harvestable': genotypes were considered harvestable if they had a visual score of 5 or less on the OIV-453 scale for bunches in July (20–30% of bunches strongly attacked). Harvestable genotypes all had a median of disease severity on bunches lower than 50%.

For powdery mildew, field assessment was not possible because of a lack of natural infection.

Statistical analyses

Powdery mildew bioassay

As inoculation was performed by blowing air onto sporulating-leaf sources on top of the tower, the homogeneity of inoculation was verified by comparing the sporulation level on the susceptible discs located in each Petri dish. A variance analysis was carried out on sporulation cm^{-2} of the susceptible control Cabernet Sauvignon discs from cuttings (one disc/Petri dish, nine Petri dishes/inoculation tower). The effects of combinations of resistance (QTL effect) on sporulation were then assessed in an analysis of variance with one factor (QTL) (general linear modelling procedure in SAS). Disease severity was log-transformed to homogenize the variance. The level of sporulation of QTL was compared by a Tukey's multiple comparison test.

Downy mildew bioassay

A non-parametric Kruskal–Wallis test was used to test the effect of the combination of resistances (QTL effect) on the OIV score for resistance.

Field experiments

The effect of combinations of resistance (QTL effect) on downy mildew severity on leaves and bunches was assessed by an analysis of variance with one factor (QTL). Data were averaged for the four repetitions of each genotype. Genotypes having the same QTL combination were considered as replicates within QTLs. Severity on leaves was square-root-transformed to homogenize the variance. Graphs of residuals were examined and Shapiro–Wilk tests were performed to check the model assumptions.

The relationship between the average severity on leaves and that on bunches was also tested by Pearson's correlation test.

Relationship between bioassays and field experiments

To perform the ROC analysis, genotypes were divided into two groups based on the condition of bunches in the field in September: harvestable (considered as 'healthy' or 'acceptable') or non-harvestable ('diseased' or 'not acceptable'). Each genotype was also assigned into an OIV-452 class based on its behaviour in the leaf bioassay ([0:1],]1:3],]3:5],]5:7]). For each OIV threshold (≤ 1, ≤ 3, ≤ 5, >5), the following were calculated: the true positive proportion (TPP), the true negative proportion (TNP), the false negative proportion (FNP) and the false positive proportion (FPP) (Table 1). The accuracy (TPP + TNP) and power (1– (1–sensitivity)) of the tests were calculated to determine the frequency of correctly classified individuals (true positive and true negative) and the probability that the test would reject a false null hypothesis (i.e. reject the non-harvestable genotypes). The ROC curve showed the relationship between true positive proportion and the false positive proportion across all possible threshold values of a predictor (i.e. OIV-452 score). TPP thus provided an estimate of the conditional probability of obtaining an OIV score below or equal to the determined threshold value, given that the true status of a genotype was non-harvestable, $(OIV \leq OIV_{thresh}|NH)$. TNP provided an estimate of the probability of obtaining an OIV score above the determined threshold value, given that the true status of a genotype was harvestable, $(OIV > OIV_{thresh}|H)$. With the ROC curve, a plant breeder can evaluate the consequences of adopting a particular threshold OIV score for the selection of grapevine genotypes. The chosen threshold for discarding genotypes may modify the sensitivity and specificity of the test. Youden's index (J = sensitivity + specificity) was calculated to identify the threshold providing the maximum accuracy for predicting bunch resistance on the

Table 1 Diagnostic test for assessing the reliability of a bioassay on leaves for predicting disease severity on bunches

	Condition = genotypes with high damage on bunches in the field	
OIV value of leaf bioassay	True = non-harvestable	False = harvestable
Low (susceptibility)	TPP[a]	FPP[a] (type I error)
High (resistance)	FNP[a] (type II error)	TNP[a]
	Sensitivity[b] = TPP/True	Specificity[b] = TNP/False

[a]TPP: true positive proportion, proportion of genotypes with a low value in the OIV test which were non-harvestable in the field; FPP: false positive proportion, proportion of genotypes with a low score in the OIV test which were harvestable; FNP: false negative proportion; TNP: true negative proportion.

[b]Sensitivity: measure of how well the binary classification test correctly identified the non-harvestable genotypes; specificity: measure of how well the test identified the harvestable genotypes.

basis of observed resistance in leaf bioassays. Bayes' theorem was used to calculate the probability of selecting a resistant genotype that would also show bunch resistance in the field (harvestable), on the basis of a positive test result for leaves in the bioassay ($H|OIV > OIV_{thresh}$) and of the proportion of resistant genotypes in the screened population (prior probability).

Results

QTL assessment

For powdery mildew, the number of genotypes (including the parents) corresponding to the four combinations of QTL resistance was eight for [Run1 QTLRgP], nine for [Run1 -], 17 for [- QTLRgP] and four for [- -]. For downy mildew, the frequencies were seven for [Rpv1 QTLRgD], 10 for [Rpv1 -], eight for [- QTLRgD] and 13 for [- -]. All genotypes displayed either both the markers flanking the *Rpv1–Run1* region or neither of these markers (no recombinant genotypes). In the absence of resistance QTLs from Regent, resistance to powdery mildew was always associated with resistance to downy mildew, suggesting that the QTLs from *M. rotundifolia* (*Rpv1* and *Run1*) were linked in this cross.

Bioassays

For powdery mildew, 16 of the 36 genotypes had levels of resistance as high as that of the resistant parent 3082-1-42, showing all developmental stages from ungerminated to conidia initiation (according to Leinhos *et al.*, 1997; Fig. 1). These genotypes were all carrying the *Run1* resistance gene and genotypes with both resistance genes [Run1 QTLRgP] were not significantly different than those with only *Run1*, both being blocked anywhere from the first stage up to the conidia initiation stage. The analysis of variance based on sporulation levels showed an effect of QTL combination (d.f. = 3, $F = 164{\cdot}77$, $P < 0{\cdot}0001$). Genotypes with *Run1* displayed significant lower levels of sporulation than those without this resistance gene and, on average, genotypes with the resistance from Regent [- QTLRgP] also displayed lower levels of sporulation than genotypes with no resistance at all (Table 2; Fig. 2a). The level of sporulation of 3082-1-42 was significantly lower than that of Regent ($P = 0{\cdot}029$ according to the Mann–Whitney *t*-test) and that of the Merlot ($P = 0{\cdot}029$) and Cabernet Sauvignon controls ($P = 0{\cdot}029$), but Regent was not significantly different from the two susceptible controls.

For downy mildew, all the genotypes sporulated with a maximum OIV-452 value of 7. *Rpv1* conferred only partial resistance to downy mildew, with considerable variation in infection levels between genotypes ($3 \leq$ OIV-452 ≤ 7; Fig. 2b, Table 2). The Kruskal–Wallis test based on the OIV-452 scores showed an effect of QTL combination ($K\text{-}W_{statistic} = 24{\cdot}15$, $P < 0{\cdot}0001$). Genotypes with *Rpv1* or [- QTLRgD] were more resistant than those with no resistance genes.

Field assessment of resistance to downy mildew

QTL combination had a significant effect on disease severity on both leaves and bunches, in both 2007 and 2008 (Table 3).

On leaves, *Rpv1* combinations displayed significantly lower levels of disease than the other combinations in June and July (mean severity < 4) in both years, despite strong inoculum pressure (Fig. 3a,b). In 2007, the Regent QTL alone [- QTLRgD] gave a level of resistance intermediate between the susceptible [- -] and *Rpv1* combinations, with a mean severity on leaves of 27%. In 2008, genotypes with the Regent QTL did not differ significantly from susceptible genotypes. In September, mean severity on leaves reached 34% in 2008 for the [Rpv1 QTLRgD] resistance gene combination.

On bunches, disease levels were much higher than on leaves and the variance between QTL combinations was greater in both years (Table 3; Fig. 3c,d). Symptoms differed between susceptible genotypes, with high levels of sporulation on infected flowers or young berries (grey rot), and resistant genotypes, with mostly discoloured berries (brown rot, no sporulation; data not shown). Genotypes with both types of resistance [Rpv1 QTLRgD] displayed significantly lower levels of disease (18% severity) than QTL combinations without *Rpv1* for both years ([- -] or [- QTLRgD]), but were not significantly different from [Rpv1] alone. In 2008, genotypes with *Rpv1* displayed significantly lower disease severity than genotypes with only QTLRgD, which were not significantly different from susceptible genotypes [- -], as observed for leaves. For bunches from susceptible genotypes, mean disease severity was 75% in 2007 and 82% in 2008.

Relationships between downy mildew resistance on leaves and on bunches

In the field, a significant correlation between the average disease severity per genotype on leaves and on bunches was observed in June ($P < 10^{-4}$, $R^2 = 0{\cdot}39$ in 2007; $P < 10^{-4}$, $R^2 = 0{\cdot}52$ in 2008).

All genotypes considered harvestable in September had a disease severity of <6·7% on leaves in both years. By contrast, the parent cultivar Regent displayed 11·2% disease severity on leaves in 2007, but was nonetheless harvestable (Fig. 4a,c). All the harvestable genotypes carried the *Rpv1* resistance gene, and about half of these genotypes also carried the Regent QTL (Fig. 4b,d). However, there was no significant positive effect of the combination of *Rpv1* with the Regent QTL. Within the *Rpv1* harvestable genotypes, disease severity on bunches varied with genotype, from 1 to 48% in 2007 and from 10 to 46% in 2008. Two to five genotypes carrying *Rpv1* per year were not resistant enough to be harvestable. Both parents, 3082-1-42 [Rpv1 -] and Regent [- QTLRgD], were harvestable in 2007, whereas Regent was not

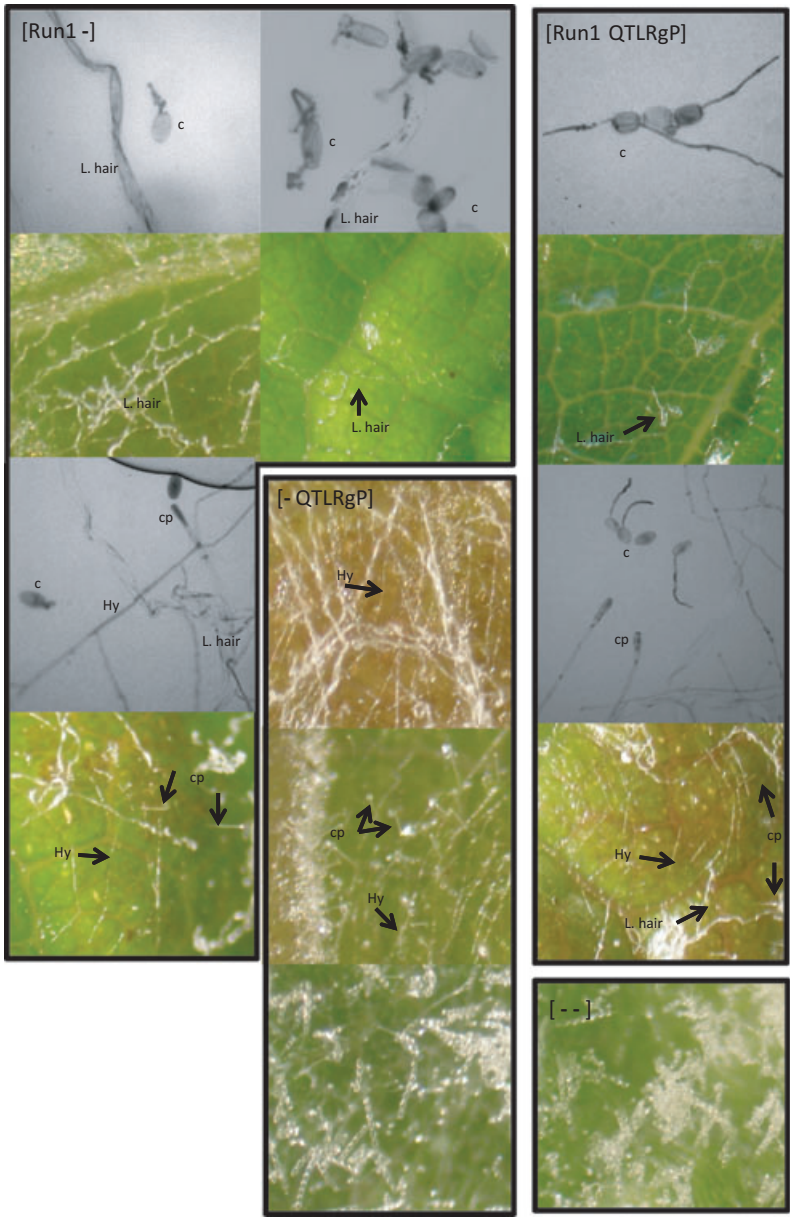

Figure 1 Interactions observed on leaf discs between different grapevine genotype combinations of *Run1* and/or QTLRgP from the population 3082-1-42 × Regent, inoculated with powdery mildew (*Erysiphe necator*). Black and white photographs are microscopic observations after a tape test and colour photographs are observations under a binocular stereomicroscope. c: germinated spores; Hy: mycelium hyphae; cp: conidiophore; L. hair: leaf hair.

harvestable in 2008. Several *Rpv1* genotypes with high levels of resistance on leaves in June (<5%) were not harvestable in September.

Reliability of bioassays for predicting resistance on bunches

In 2007, the frequency distributions of bioassay OIV-452 scores plotted separately for genotypes classified as harvestable and non-harvestable overlapped (Fig. 5a). Overall, 15% of the genotypes considered the most resistant (harvestable) were scored as intermediate to susceptible according to the OIV scale in the bioassay (≤5), and 5% of genotypes considered resistant in the bioassay (>5) were not harvestable in the field. Despite these errors in the prediction of field disease resistance on the basis of bioassay results, the ROC curve analysis demonstrated that the bioassay provided a useful indication of field resistance (Fig. 5b); the area under the ROC curve was 0·86. An OIV threshold of five was found to

Table 2 Average sporulation and OIV score for each combination of QTLs and control grapevine taxa based on the results of bioassay tests of resistance to powdery (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*), respectively

Powdery mildew					Downy mildew				
Gene combination[a]	$N_{genotypes}$	N_{obs}	Average sporulation	SE	Gene combination[b]	$N_{genotypes}$	N_{obs}	Average OIV	SE
[Run1 RgP]	8	19	1430	200	[Rpv1 RgD]	7	7	5·8	0·699
[Run1 -]	9	24	1659	309	[Rpv1 -]	10	10	5·1	1·483
[- RgP]	17	67	42 730	3641	[- RgD]	8	8	4·2	1·510
[- -]	4	16	75 340	8594	[- -]	13	13	1·8	0·832
Regent		4	40 400	10320	Regent		6	3·0	0·000
3082-1-42		3	1555	312	3082-1-42		5	6·7	0·330
Merlot		4	59 610	23420	Merlot		6	2·3	0·422
Cabernet Sauvignon		4	63 860	5298					

[a][Run1 RgP]: *Run1* resistance gene + QTL of Regent; [Run1 -]: *Run1* resistance gene only; [- RgP]: QTL of Regent only; [- -]: no resistance gene.
[b][Rpv1 RgD]: *Rpv1* resistance gene + QTL of Regent; [Rpv1 -]: *Rpv1* resistance gene only; [- RgD]: QTL of Regent only; [- -]: no resistance gene.

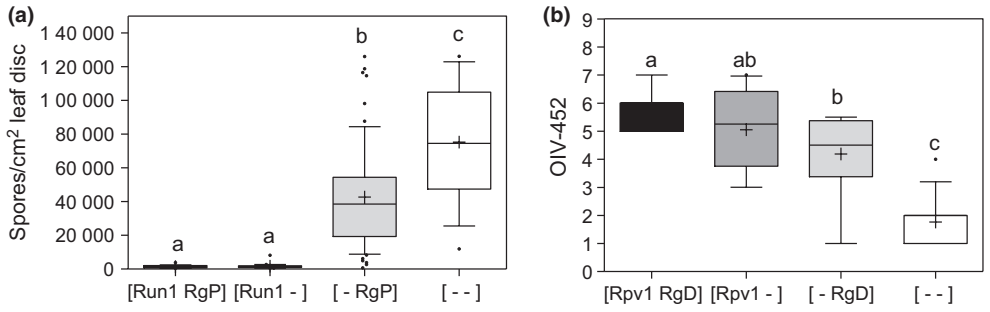

Figure 2 Box-whisker plots of level of infection (OIV-452) or of sporulation on leaf discs in the laboratory, for the various grapevine genotypes, as a function of the combination of resistance genes. (a) powdery mildew (*Erysiphe necator*); (b) downy mildew (*Plasmopara viticola*). Whiskers exclude values <10th percentile and >90th percentile. Outliers beyond whiskers are shown as points and mean is indicated in the box as +. Different letters above the plots indicate a significant difference at $P < 0.05$ according to Tukey's multiple comparison test (for powdery mildew) or Dunn's multiple comparison test (for downy mildew).

Table 3 Analysis of variance for the severity of downy mildew (*Plasmopara viticola*) on bunches and on leaves, in the vineyard in 2007 and 2008

			Severity on leaves			Severity on bunches		
Year	Source of variation	d.f.	Mean square	F_{value}	$P_{value} > F$	Mean square	F_{value}	$P_{value} > F$
2007	Model	3	101·9	64·3[a]	<0·0001	5708·4	14·7	<0·0001
	Error	34	1·6			388·5		
2008	Model	3	53·1	67·4	<0·0001	6572·8	25·3	<0·0001
	Error	34	0·8			259·4		

[a]Variable transformed as square root.

give the maximum value for Youden's index. At this threshold, for the tested population, the sensitivity (i.e. the probability of the bioassay correctly identifying non-harvestable genotypes) was 0·91, the specificity (the probability of the bioassay correctly identifying the harvestable genotypes) was 0·63 and the overall test accuracy was 0·79 (Fig. 5c). The power of the test (i.e. the probability of rejecting a non-harvestable genotype) was high (0·91). For this threshold, the likelihood ratio was 2·42, i.e. non-harvestable genotypes were 2·42 times as likely to be discarded as harvestable genotypes. Based on this likelihood ratio, the probability of selecting a resistant (harvestable) genotype based on its having an OIV score > 5 in the bioassay was calculated for a new population (Fig. 5d). For a population with 0·42 resistant genotypes [Rpv1] (prior probability), the probability of selecting a genotype resistant in the field, based on OIV tests with a threshold score of 5, was 0·83. For a population tested earlier in the selection process with a lower prior probability, the probability of selecting resistant

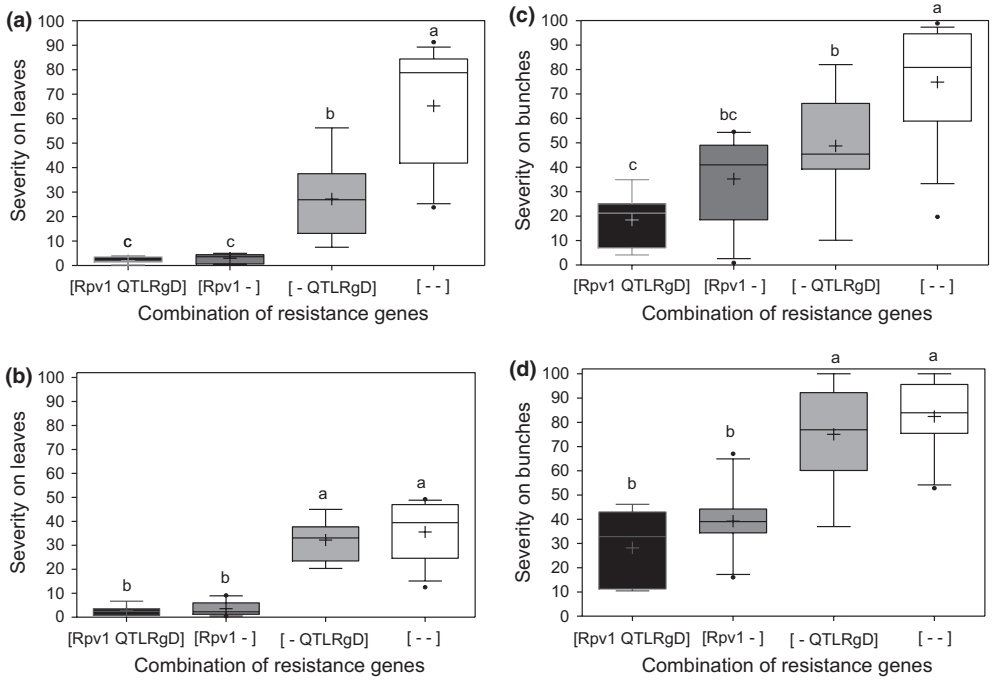

Figure 3 Box-whisker plots of severity of downy mildew (*Plasmopara viticola*) on leaves (a, b) and on bunches (c, d) for various grapevine genotypes, in the vineyard, as a function of the combination of resistance genes. (a, c) 29 June 2007; (b, d) 7 July 2008. Whiskers exclude values <10th percentile and >90th percentile. Outliers beyond whiskers are shown as points and mean is indicated in the box as +. Different letters above the plots indicate a significant difference at $P < 0.05$ according to Tukey's multiple comparison test.

genotypes based on OIV score would be much lower. With this threshold, cv. Regent (OIV = 3) would not have been selected. ROC curve analysis for 2008 identified the same OIV score threshold (OIV = 5) for selecting genotypes with acceptable disease resistance on bunches (accuracy = 0·84, specificity = 0·75, sensitivity = 0·88). Similarly, based on the bioassay, it is possible to select with good accuracy a genotype highly resistant on leaves in the field (severity ≤ 5%) based on an OIV score > 3.

Discussion

This study combined data from bioassays with data from field experiments to explore the resistance to disease caused by pathogens of a major perennial crop, grapevine. Using this approach, it was possible to assess the reliability of bioassay tests for predicting resistance level to downy mildew on adult plants under field inoculation conditions. The performance and variability of QTLs for resistance were also evaluated and the ability of bioassays on leaves to predict resistance on bunches in the field based on a Bayesian framework was assessed.

For powdery mildew, the bioassays performed on young leaves of the same age taken from the field allowed unambiguous discrimination between genotypes carrying *Run1* and genotypes lacking this resistance gene. *Run1*-carrying genotypes displayed total to high levels of resistance, whereas genotypes without *Run1* displayed intermediate to high levels of sporulation. Although *Run1* is very effective, the observation of some sporulation and mycelium growth on some genotypes may suggest that the development of the pathogen is possible. This is in line with recent observations made in North America of the presence of the disease and even of cleistothecia on *Run1* genotypes in an experimental vineyard (Cadle-Davidson *et al.*, 2011). In the present study it was not possible to evaluate the relationship between the bioassay and field resistance of bunches because the disease did not occur in the vineyard during the 2 years of experimentation. Investigation of this relationship will require the successful artificial inoculation of bunches at the correct phenological stage, in order to control epidemics and ontogenic resistance.

Rpv1 confers a partial level of resistance to downy mildew. In the bioassay, considerable variability between genotypes was observed, with some genotypes as susceptible as Regent, whereas in the field, resistance resulted in lower disease severity scores on leaves in June, with lower level of variation between genotypes. Indeed, among the 47% of genotypes expressing a high level of resistance on leaves in the field (<5% severity), only 26% were identified as highly resistant (]5:7]) in the bioassay (cf. Fig. S1). The observed differences between the behaviour of *Rpv1* in the field and in bioassays may result from differences in tissue susceptibility for mature leaves. One hypothesis is that minor genes present in *Rpv1* genotypes are expressed in the field and enhance *Rpv1* resistance, restricting the

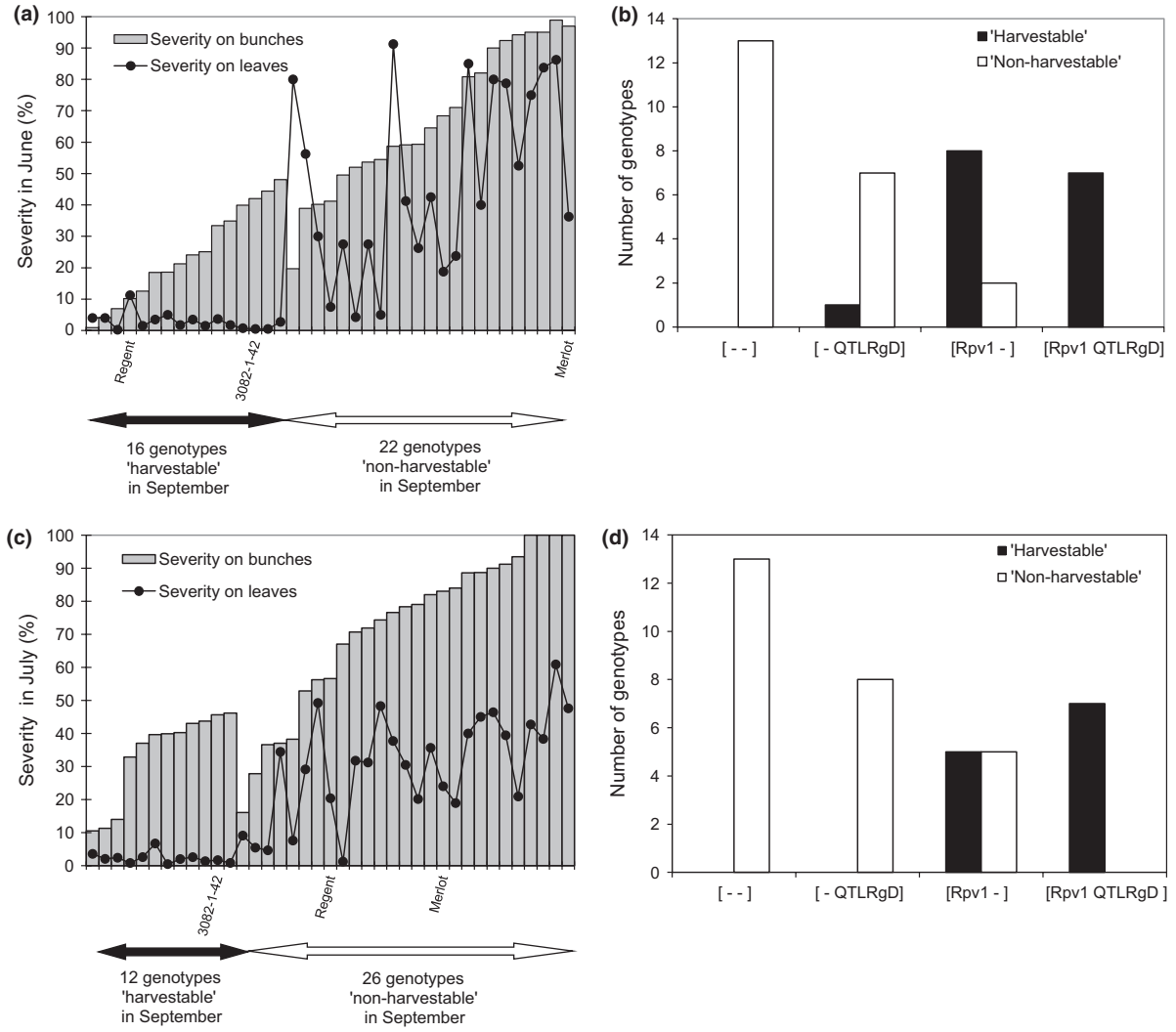

Figure 4 Distribution of downy mildew (*Plasmopara viticola*) disease severity on leaves and bunches in the field in June 2007 (a) and 2008 (c) for various grapevine genotypes considered harvestable and non-harvestable in September 2007 (b) and 2008 (d), with their classification into the four QTL combinations.

extension of symptoms. Bioassays carried out at high inoculum pressures on susceptible leaves from cuttings could underestimate the actual level of resistance of *Rpv1* genotypes expressed in the field. The level of inoculum used in bioassays should therefore be varied to identify the bioassay scores most closely matching the observed behaviour of *Rpv1* in the field.

The Regent QTLs displayed a highly variable level of resistance in bioassays to both diseases: sporulation varying by a factor of 7 for powdery mildew, and OIV score varying from 1 to 5·5 for downy mildew. Regent itself was classified as susceptible, with an OIV score of 3. In the field, resistance ranged from intermediate levels in 2007 to total susceptibility in 2008. Despite the identification of the QTLs associated with downy mildew resistance in Regent (Fischer *et al.*, 2004; Welter *et al.*, 2007) and the ability of this variety to up-regulate genes with possible roles in plant defence (Figueiredo *et al.*, 2008), this resistance seems to be unstable. The main hypothesis to explain these results is that *P. viticola* populations have undergone a modification of their virulence and/or aggressiveness. It is worth noting that Kast *et al.* (2000) demonstrated that downy mildew isolates collected on Regent could show increased aggressiveness on this variety. In addition, although data on virulence of *P. viticola* is scarce, Peressotti *et al.* (2010) showed that resistance-breaking isolates of *P. viticola* overcoming monogenic resistance can arise even for resistant varieties cultivated over limited geographic areas. It can also be hypothesized that climatic differences between years had an impact on the development of the varieties, with better synchronization between host development and the onset of the epidemic.

For genotypes carrying *Rpv1,* resistance was weaker and more variable on bunches than on leaves. Not all *Rpv1* genotypes were harvestable, but all harvestable

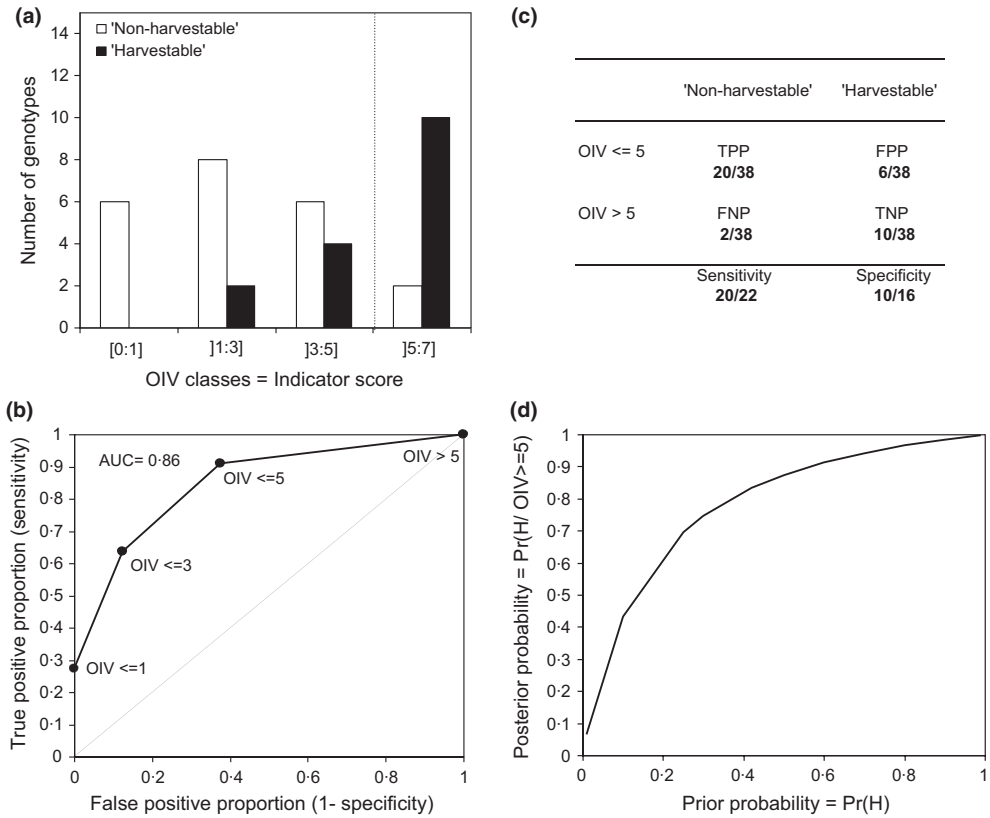

Figure 5 Frequency distribution of bioassay indicator scores (OIV classes) for grapevine genotypes characterized as non-harvestable and harvestable in the field (a), the corresponding ROC curve (b), distribution of genotypes depending on their characterization on bunches and their bioassay indicator scores (c), and predicted selection of a resistant harvestable genotype given a score of at least 5 in the OIV test (d). TPP: true positive proportion, the proportion of genotypes with a low OIV score that were non-harvestable; FPP: false positive proportion, genotypes with a low OIV value that were harvestable; TNP: true negative proportion, genotypes with a high OIV value that were harvestable; FNP: false negative proportion, genotypes with a high OIV value that were non-harvestable.

genotypes possessed *Rpv1*. There are several possible explanations for the variability of resistance in bunches: (i) the resistance gene may be expressed differently in bunches; (ii) the resistance gene may not be expressed in bunches, with the observed resistance of bunches instead being caused by variations in ontogenic resistance (age-related resistance) between genotypes; and/or (iii) the variability of resistance may be linked to differences in disease dynamics on leaves or to differences in the amount of primary inoculum released from the soil.

Some of the genotypes carrying *Rpv1* may differ in the timing and dynamics of the development of ontogenic resistance in bunches and/or in the effects of developmental factors on the expression of resistance. Disease resistance may be modulated by the general development of the plant. Ontogenic resistance is widespread in plants (Develey-Rivière & Galiana, 2007) and its expression is well documented for the main diseases of vine: black rot disease (Molitor & Berkelmann-Loehnertz, 2011), botrytis (Salzman *et al.*, 1998), powdery mildew (Ficke *et al.*, 2003) and downy mildew (Kennelly *et al.*, 2005). For powdery mildew, ontogenic resistance has been observed on bunches and varies with *Vitis* species (Gee *et al.*, 2008). For downy mildew, the susceptibility and symptoms of bunches also vary with berry development, cultivar, site and year (Kennelly *et al.*, 2005). In the field observations in the present study, diseased bunches from *Rpv1* genotypes displayed discoloured berries, consistent with inoculation at late developmental stages, after the conversion of functional stomata to lenticels, preventing the pathogen from emerging through the stomata and sporulating. Kennelly *et al.* (2005) showed that the time of ontogenic resistance onset in fruit clusters varied among cultivars of *V. vinifera* and *V. labrusca*. The variability of severity on bunches between and within *Rpv1* genotypes may also be increased by differences in the sensitivity of flower development to climate. Thus, bunches must be inoculated at different developmental stages if understanding of the interaction between disease resistance and bunch development is to improve. This step is required to increase the durability of resistance, thereby extending the use of the resistant cultivars and cropping systems best suited to the environmental conditions. In

the future, the measurement of phenological traits together with analyses of molecular markers should make it possible to identify QTLs associated with the factors increasing disease resistance in bunches. It is worth noting that QTLs associated with morphological traits have already been identified in preliminary studies of morphogenetic regulation in grapevine (Welter *et al.*, 2007). The few measures of host growth performed in 2007 in the present study showed a significant correlation between leaf phenology in April (number of leaves/shoot) and bunch severity in June (R^2 = 0·23). Correlation between the earliness of budbreak and the development of bunches is expected. To assess the contribution of the different factors on bunch severity, partial least square path modelling (Tenenhaus *et al.*, 2005) was performed. Although the results could be refined with more variables, the contributions of QTLs, disease on leaves and host growth variables to disease variability on bunches were evaluated as 44, 40 and 14%, respectively. These three variables allowed 58% of disease variation to be explained (cf. Fig. S2). This means that about half of the variation of disease severity on bunches can be explained by a decrease of inoculum on leaves, but half could not and was linked to the direct expression of the QTL resistance on the bunches. There was no significant effect of genotype position in the plot. The unexplained variability may result from the heterogeneity of primary infections. Indeed, bunches may be contaminated by infected leaves or directly by oospores released from the ground.

In this study, a generic approach was applied to decision-making processes in breeding for disease resistance. Bayesian decision theory provides a framework for objective decision-making (e.g. selecting a genotype) and for evaluating decisions that have been made. This method has long been used in medical research for the evaluation of indicators linked to human diseases that could be used for diagnostic purposes (Metz, 1978). It has recently been applied to plant disease management for the prediction of disease outbreaks, the evaluation of disease predictors and the validation of forecasting models (Turechek & Wilcox, 2005; Caffi *et al.*, 2011). The present study evaluated whether a bioassay performed on leaves at an early stage of selection was a relevant indicator of resistance to downy mildew on bunches in the field. The Bayesian decision analysis was used to determine the probability of selecting harvestable genotypes using the bioassay resistance rating as a predictor, as a function of the frequency of resistant genotypes in the population. Analyses of response curves showed that bioassays, as currently performed by grapevine breeders, are a suitable tool for predicting satisfactory behaviour of bunches in the field, providing a high downy mildew resistance level threshold is used as the selection criterion in the bioassay. The identification of a threshold (OIV > 5) maximizing the chances of the plant yielding harvestable bunches is of particular interest at several critical steps of a breeding programme. Because it saves time and space, it could be systematically applied during the intensive screening of genetic resources. The results obtained here are promising, providing support for the hypothesis that when a high level of resistance is expressed in leaves, the resistance genes responsible are very likely to be expressed in bunches too (in addition to the reduced inoculum pressure coming from the leaves). However, confirmation on a wider range of crosses and for other resistance QTLs is required.

Finally, the questions of the performance of the resistance genes studied and of their durability in the long term remain to be addressed. As shown by the variability of genotypes, the pyramiding of resistance genes may not always be efficient. Moreover, some of the genotypes with effective resistance to downy and powdery mildew may be highly susceptible to other diseases, such as black rot, as for the *M. rotundifolia* parent. Trade-offs between resistances to disease in grapevine can exist, as reported for some varieties of barley in which *mlo* resistance depends on genetic background (Pinnschmidt *et al.*, 2007). These results concerning the expression of resistance, together with previous knowledge about the mechanisms of resistance (Unger *et al.*, 2007; Diez-Navajas *et al.*, 2008), underline the need to pyramid major resistance genes with genes that have minor effects for several diseases to increase the durability of these resistances.

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Supporting Information

Additional Supporting Information can be found in the online version of this article:

Figure S1. Frequency distribution of bioassay indicator scores (OIV classes) for genotypes characterized for their level of severity on leaves (<5% or ≥5%) in the field.

Figure S2. PLS path model describing the relationships between the endogenous latent variable 'disease on leaves', 'disease on bunches' to the other latent exogenous variables 'host growth' and 'QTL'.

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