Diversity and Fitness of *Plasmopara viticola* isolates resistant to QoI fungicides

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Effective control measures for downy mildew caused by the oomycete *Plasmopara viticola* include Quinone outside inhibitor fungicides (QoIs). However, strong selection pressure following repeated QoI applications may result in the development of QoI-resistant populations, and this has limited the efficacy of these fungicides in grapevine (Grasso *et al.*, 2006). Only a few years after the introduction of QoI fungicides, *P. viticola*-resistant populations were detected throughout European and United States vineyards (Baudoin *et al.*, 2008; Corio-Costet *et al.*, 2008; Sierotzki *et al.*, 2005).

In most pathogens, two major mutations in the cytochrome b gene have been reported (G143A, F129L) to be involved in resistance mechanisms, with the G143A mutation most widely recognized (Gisi *et al.*, 2002). Chen *et al.* (2007) investigated the mechanism underlying the evolution of QoI resistance, by carrying out a phylogenetic analysis of a large mitochondrial DNA fragment including the cytochrome b gene (2.281 bp) across a wide range of *P. viticola* isolates. Four major haplotypes belonging to two distinct genetic groups (I and II) were identified, each of which contained a different QoI fungicide-resistance allele carrying the G143A mutation (Chen *et al.*, 2007). These findings thus indicated that there were at least two origins of fungicide resistance in grapevine downy mildew populations. In France, group I (IR and IS) and group II (IIR and IIS) haplotypes have reached a mean frequency of 75% and 25% respectively, but their distribution may differ between regions (Corio-Costet *et al.*, 2008; Chen *et al.*, 2007). The resistant haplotype IR has been found to account for 67.7 to 98.3% of the resistance alleles in the population, depending on the site considered (Chen *et al.*, 2007, Corio-Costet *et al.*, 2006).

One challenge facing researchers trying to understand the evolution of fungicide resistance is fitness measurements, assessing the selective value of the pathogen (Antonovics, and Alexander, 1989; Pringle and Taylor, 2002). The rapidity with which QoI resistance has appeared and the behavior of resistant isolates in populations suggest that the fitness of resistant isolates may be high. We assessed this here. We performed fitness and competitiveness studies to investigate the spread and maintenance of *P. viticola* and we assessed i) the diversity of QoI haplotypes in European countries, ii) the fitness of sensitive and resistant isolates by comparing latent period, sporulation, infection frequency and competitiveness and iii) the cost of QoI resistance.

Material and Methods

A total of 1366 downy mildew lesions on *Vitis vinifera* were collected between 2000 and 2004 (table 1). For isolates collection, a total of 11 QoI-sensitive isolates and 12 QoI-resistant isolates were collected mainly in 2003 at the beginning of the growing season on leaves in Bordeaux vineyard and one in Germany (table 2).

Table 1: Mean mitochondrial haplotype distribution in European populations of *Plasmopara viticola*

Country	N	% of European mitochondrial haplotypes			
		IS	IR	IIS	IIR
France	1015	56.45	20.99	20.3	2.26
Germany	93	73.12	8.6	18.28	0
Greece	21	90.47	0	9.53	0
Italy	41	85.37	0	14.63	0
Portugal	68	79.41	2.94	17.65	0
Romania	67	97.02	0	2.98	0
Switzerland	61	80.33	0	19.67	0
Europe (Mean ± Sem)	1366	**80.19 ± 4.98**	**4.14 ± 2.91**	**15.18 ± 2.56**	**0.5 ± 0.5**

To characterize the sensitivity of *P. viticola* isolate to QoI fungicides, single lesions were multiplied onto leaves of *Vitis vinifera*. Inoculation was performed as previously described (Chen *et al.*, 2007; Corio-Costet *et al.*, 2006). The QoI tested was famoxadone (3-anilino-5-methyl-5-(4-phenoxyphenyl)-1, 3-oxazolidine-2, 4-dione). A discriminatory dose of 10 mg/l, was used to determine whether a strain was resistant or sensitive. Ranges of concentrations (from 0 to 1000 mg/l for resistant strains and from 0 to 2 mg/l for sensitive strains) were used to determine the ED_{50} for each isolates Fungicide assay were carried out as previously described (Corio-Costet *et al.*, 2008, Corio-Costet *et al.*, 2010).

We measured the aggressiveness components, by quantifying thee latent period, sporulation and infection frequency as previously described (Chen *et al.*, 2007), on three plates each, containing 5 leaf discs inoculated with three droplets of a suspension of 2500 sporangia/ml. The latent period (LP) was estimated by monitoring the daily development of sporangia and determining the mean time required to obtain 50% sporulation. Spore production (Nt/Nt0) was assessed by washing the five inoculated discs from each plate in a vial with Isoton solution and determining the number of sporangia produced per plate in a Coulter multisizer counter. Infection frequency (IF) was defined as the proportion of inoculated leaf discs on which lesions developed 7 days after inoculation. A composite fitness index (Fi) was then calculated from the formula $Fi = Ln (N_t / N_{t0} \times IF \times 1/LP)$ (Corio-Costet *et al.*, 2010).

The relative competitiveness of two pairs of resistant/sensitive isolates R1-S1 and R2-S1 was compared at three initial R:S ratios of spores concentrations: 20:80, 50:50 and 80:20. Mixed inocula were generated by mixing such that the final suspension contained 40,000 spores per ml. Four plates, each containing 5 discs, were inoculated. We then followed a series of eight consecutive asexual cycles. We quantified the proportion of resistant isolates after each asexual cycle, by carrying out a biological test with 100 mg/l famoxadone, as described above, and a QoI real time Q-PCR quantification with specific primers for

quantification of the *Cyt b* gene and the resistant allele described by Sirven *et al.* 2002 (Sirven *et al.*. 2002).

Results

The SNP typing assay was used to survey the geographical distribution of various mitochondrial haplotypes from a panel of 1366 *P. viticola* isolates. The frequency of the four main European haplotypes was estimated (Table 1). Haplotype group I (IS and IR) predominated in all countries, accounting 77.44 to 97.02% (mean 84.33%). Haplotype II (IIS and IIR) accounted for 2.98 to 22.56% depending on the countries, with mean 15.68%. Haplotype IR predominated among strains resistant to QoIs, as expected accounting for 0 to 20.99% (mean 4.14%)of strains. The resistant haplotype IIR was the least common.

We evaluated the sensitivity to QoIs of 11 isolates classified as sensitive and 12 isolates classified as resistant collected in 2003 (Table 2). Sensitive isolates had ED_{50} values of 0.1 to 0.9 mg/l whereas those for resistant isolates exceeded 1000 mg/l. Seven sensitive isolates belonged to haplotypes II, the remaining four belonging to haplotype I. By contrast, 8 resistant isolates belonged to haplotype I, whereas only 4 belonged to haplotype II. The mean relative resistance factor between resistant and sensitive was 5263.

Table 2: Origin and characteristics of monosporangial isolates used in fitness studies

Isolate[1]	Locality of vineyard	Year	Mitochondrial haplotype (I or I)	QoI sensitivity	QoI CI_{50} (mg/ml)
S1	Bordeaux	2003	I	S	0.07
S2	Champagne	2003	II	S	0.90
S3	Bourgogne	2003	II	S	0.10
S4	Bordeaux	2003	II	S	0.05
S5	Bordeaux	2003	II	S	0.10
S6	Bordeaux	2003	II	S	0.07
S7	Champagne	2003	I	S	0.22
S8	Bordeaux	2003	II	S	0.13
S9	Bourgogne	2003	II	S	0.04
S10	Rhône Valley	2003	I	S	0.09
S11	Alsace	2003	I	S	0.28
R1	Midi-pyrénées	2003	I	R	>1000
R2	Bordeaux	2003	II	R	>1000
R3	Bordeaux	2003	II	R	>1000
R4	Midi-pyrénées	2003	I	R	>1000
R5	Freiburg	2003	I	R	>1000
R6	Midi-pyrénées	2003	I	R	>1000
R7	Bordeaux	2003	I	R	>1000
R8	Bordeaux	2003	I	R	>1000
R9	Bordeaux	2003	I	R	>1000
R10	Bordeaux	2003	I	R	>1000
R11	Bordeaux	2003	II	R	>1000
R12	Bordeaux	2003	II	R	>1000

Data on the fitness of the 23 isolates inoculated separately (Table 3) showed there to be no difference (P= 0.523) in latent period between sensitive and resistant isolates. The latent period was between 86.5h to 140h for sensitive isolates and 89.5 to 139h for resistant isolates.

No significant difference in spore production was observed between sensitive and resistant isolates (P= 0.137). Sensitive isolates produced a mean of 704 ± 139 sporangia and resistant isolates produced 830 ± 171 sporangia per deposited sporangium (Table 3). Considerable variation was observed within the sensitive isolate group and the QoI-resistant group. In our experimental conditions, there was a significant difference in infection frequency between sensitive and resistant isolates with 0.84 ± 0.035% of sensitive isolates and 0.94 ±0.02% of resistant isolates successfully infecting leaves (P=0.02). IF was between 0.42 and 0.97 in the sensitive group and between 0.68 and 1 in the resistant group.

A composite fitness index was calculated for each isolates based on the F_i of Tooley (1986). Mean Fi was 1.75 ± 0.65 for sensitive group and 2.05 ± 0.45 for the resistant group, but this difference between the groups was not significant (P=0.147). The fitness index varied considerably depending on whether the isolates were sensitive or resistant. The Fi value obtained was then used in the selection of sensitive and resistant isolates for competitiveness tests.

Table 3: Fitness components of sensitive and QoI-resistant isolates on grapevine leaf disks at 22°C (means ± SEM).

Isolates	QoI Sensitivity	Latent Period (h ± SEM)	Sporulation[1] (Nt/N_{t0}) (± SEM)	Infection Frequency (± SEM)	F_i (Ln (Nt/N_{t0} IF /LP)
11 sensitive	S	**102.7 ± 1.57**	**704 ± 139**	**0.84 ± 0.035**	**1.75 ± 0.65**
12 resistant	R	**100.9 ± 1.32**	**830 ± 171**	**0.94 ± 0.02**	**2.05 ± 0.45**

[1]: Sporulation is expressed as the ratio of spores produced per deposited spore after 7 days of growth ± SEM.

The objective was to assess the competitiveness of resistant and sensitive isolates in mixed-isolate inoculations on leaf disks and to link these findings with the fitness parameters of isolates. Changes in the frequency of resistant isolates were largely dependent on the competing isolates and on their initial fitness index. In figure 1, the sensitive isolate S1 had an Fi value of 1.78, a figure typical of sensitive isolates (mean 1.75, Table 3). The resistant isolate R1 with which it was mixed (figure 1) had an F_i of 2.15 close to the mean value for resistant isolates. In the second pair tested, we mixed an S1 with an R2 isolate (figure 1), which had an Fi (1.57) lower than the Fi of the S1 isolate. After eight asexual cycles all ratios between sensitive and resistant isolates remained stable at 20, 50, and 80% R1, after quantification by biological assays and Q-PCR. A small but significant increase (P< 0.05) from the initial ratios of 20% and 50% was observed in the eighth cycle. In the second pair (figure 2), a decline in the frequency of R2 was observed after five cycles, for all three initial ratios, indicating that the resistant isolate R2 competed only weakly with its S1 partner.

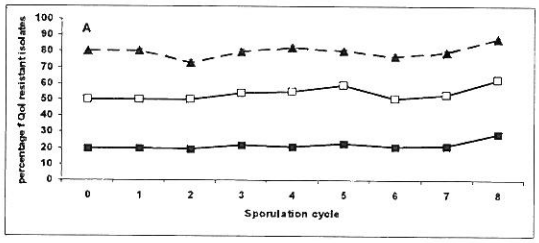

Figure 1: Dynamic changes (means ± SEM) in the frequency of QoI-resistant isolates in the sporangial populations harvested from grapevine leaf disks inoculated with three mixtures of QoI-sensitive S1 and QoI-resistant sporangia R1 in various proportions (20:80 (-■-), 50:50 (-□-) and 80:20 (--▲--), with monitoring over eight asexual generations. The second sporulation cycle was initiated with sporangia resulting from the initial infection and subsequent sporulation cycles were

initiated in a similar manner. Each point is mean of six replicates. **A**: assessment of QoI-resistant sporangia by biological tests

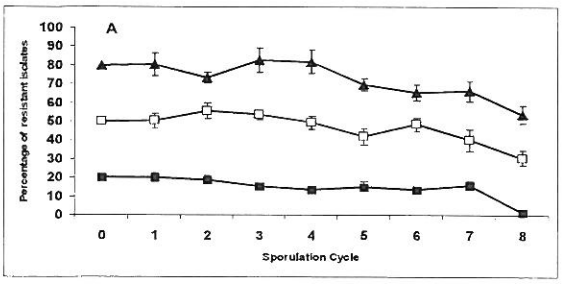

Figure 2: Dynamic changes (means ± SEM) in the frequency of QoI-resistant isolates in sporangial populations harvested from grapevine leaf disks inoculated with three mixtures of QoI-sensitive S1 and QoI-resistant sporangia R2 in various proportions (20:80 (-■-), 50:50 (-□-) and 80:20 (--▲--), over eight asexual generations.

Conclusion

Previous results for French vineyards (Corio-Costet *et al.*, 2008; Chen *et al.*, 2007, Corio-Costet *et al.*, 2006), showed haplotype1 to be the most widespread in Europe accounting for a mean of 84.33%. Only 4.54% of the 1366 isolates collected between 2000 and 2004 were QoI-resistant (IR, IIR), with haplotype IR accounting for 91.19% of resistant haplotypes. The role of mitochondrial haplotype I and II in the *P. viticola* population remains unclear, although it has been shown that QoI resistance in downy mildew in Europe has at least two different origins (6). It is clear that, at the beginning of the growing season, before fungicide selection pressure, most isolates are sensitive to QoI after the sexual reproduction cycle.

One of the most important factors affecting the evolution of fungicide resistance is the fitness of resistant isolates. In our study the fitness parameters of QoI-sensitive and resistant field isolates of *P. viticola* show that i) resistant isolates are as fit as sensitive isolates, in terms of sporangium production, latent period duration and infection frequency in asexual cycles, ii) QoI resistant isolates may compete successfully compete with sensitive isolates in mixed inoculation if their fitness index is sufficiently high.

No cost of QoI resistance was detected in our experimental conditions. There may be three reasons for this i) QoI resistance due to a single mutation (G143A) may have no cost, ii) there may be a cost but selection acts on resistant isolates that initially have a very high fitness level, so the cost is not detectable, or iii) there is a cost, but additional compensatory mutations or regulations restore the fitness of resistant strains.

In the case of QoI resistance, mitochondrial heritability and the frequency of sexual reproduction also determine the likelihood of the resistant allele spreading. In agriculture, a better understanding of fungal evolution might improve active manipulation of the evolutionary process, making it possible to reduce fungicide resistance in natural *P. viticola* populations.

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