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Assessment of fungicide resistance and pathogen diversity in *Erysiphe necator* using quantitative real-time PCR assays

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Abstract

BACKGROUND: Management of grapevine powdery mildew *Erysiphe necator* Schw. requires fungicide treatments such as sterol demethylation inhibitors (DMIs) or mitochondrial inhibitors (QoIs). Recently, reduction in the efficacy of DMIs or QoIs was reported in Europe and the United States. The aim of the present study was to develop real-time qPCR tools to detect and quantify several *CYP51* gene variants of *E. necator*: (i) A versus B groups (G37A) and (ii) sensitive versus resistant to sterol demethylase inhibitor fungicides (Y136F).

RESULTS: The efficacy of the qPCR tools developed was better than the CAPS method, with a limit of 2 pg for *E necator* DNA, 0.06 ng for genetic group A and 1.4 ng for the DMI-resistant allele. The detection limits of qPCR protocols (LOD) ranged from 0.72 to 0.85%, and the quantification limits (LOQ) ranged from 2.4 to 2.85% for the two alleles G47A and Y136F respectively. The application of qPCR to field isolates from French vineyards showed the presence of DMI-resistant and/or QoI-resistant alleles in French pathogen populations, linked to genetic group B.

CONCLUSION: The real-time PCR assay developed in this study provides a potentially useful tool for efficient quantification of different alleles of interest for fungicide monitoring and for population structure of *E. necator*.
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Keywords: biotype; DMI fungicide; powdery mildew; QoI; Q-PCR; resistance

1 INTRODUCTION

Grapevine powdery mildew caused by *Erysiphe necator* Schw., an obligate parasite, is one of the most widespread diseases of grapevine (*Vitis vinifera* L.) worldwide. In European and Australian vineyards, two genetic groups of *E necator*, A and B, have been distinguished.[1,2] The distribution and epidemiological significance of the two groups are unknown.[3–7] Based on the overwintering modes, groups A and B may be responsible for the primary inoculum arising from sexual reproduction and/or asexual wintering of mycelium in dormant buds.[3,8–11] In spring, at the beginning of grapevine growth, symptoms may appear in flag shoot form (asexual) or be caused by ascospore projection (sexual) in sparse plots. The different symptoms have been reported with variable spatial and temporal distribution between the beginning and the end of the growing season, and also depend on the geographical position of the vineyard.[8,10–14] Indeed, flag shoot symptoms were more frequent in Mediterranean vineyards.[7,8] As regards flag shoot symptoms at the beginning of the growing season, group A was in the majority, but group B populations were also found alone or mixed in with group A.[3,11,13] A comparison of life history traits determining aggressiveness between the two genetic groups showed that group A was less aggressive than group B in terms of the germination ratio and infection efficiency, and that group A was more aggressive than group B in terms of the latency period, lesion diameter and number of spores per lesion.[7] An association between the levels of disease severity at the end of the growing season and the initial compositions of *E. necator* populations in vineyards was previously observed,[7,8,11] and damage was found to be greater for epidemics initiated by group B isolates.[13] Until now, only group B populations have been found to give rise to sexual reproduction in France,[8,12,14–16] but some studies in Australia and Italy suggest that group A strains can also replicate by sexual reproduction.[6,17]

Erysiphe necator has been submitted to the pressure of numerous fungicides including sterol biosynthesis inhibitors (SBIs) and, in particular, the sterol C14-demethylase inhibitors (DMIs). The intensive use of DMI fungicides has led to the appearance of resistance in various countries.[8,18–21] In French vineyards the DMI-resistant isolates belong mainly to genetic group B.[8,12,22] The DMI resistance has been described as being multigenic, but with one major mechanism involving a single mutation in the

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14-eburicol demethylase gene (*CYP51*) of *E. necator*.[23,24] Indeed, in grapevine powdery mildew, the replacement of a phenylalanine residue by tyrosine at position 136 (Y136F) in the *CYP51* gene was associated with a high level of resistance to DMIs,[23] as in *Blumeria graminis* Speer.[25,26] More recently, resistance to other fungicides such as mitochondrial cytochrome b inhibitors (QoIs) has also been detected in the United States[27] and in Europe (Bencené D, private communication, 2007).

Quantitative real-time PCR (qPCR) has recently been applied to the detection and quantification of microorganisms resistant to fungicides.[27–33] Because it is a very powerful, cultivation-independent, rapid and sensitive method, it has been used to detect various organisms in a wide range of research fields.[34] It is especially interesting for non-cultivable, complex environments, and for obligate parasites such as fungi or oomycetes. QPCR methods have also been used to quantify QoI-resistant allele in *B. graminis*,[35] in *Plasmopara viticola* Berl. & De Toni[36–38] and *E. necator*,[27] resulting in a single point mutation in the mitochondrial cytochrome b, leading to the replacement of a glycine by an alanine at codon 143 (G143A). Recently, rapid quantification of DMI resistance in *B. graminis* populations has been developed.[32]

Nowadays, the tools developed to detect genetic groups or fungicide resistance in *E. necator* are PCR tools based on detection of point mutations in a specific gene such as *CYP51* coding for eburicol-C14-demethylase in sterol biosynthesis,[39] β-tubulin gene[3,13] and transposons,[40] and on the polymorphism of unknown sequences such as microsatellites (SSR), RAPD, RFLP and AFLP markers.[1,2,9,10,12,14,41,42]

In spite of the numerous studies carried out on *E. necator*, the detection and quantification of populations resistant to fungicides, as well as the role of the two genetic groups in the development of resistance in pathogen populations, remained unclear. As *E. necator* is an obligate pathogen, knowledge about how its population evolves in the vineyard is difficult to acquire because each symptom must be analysed individually with the PCR tools that currently exist. It is therefore difficult to obtain population data on a large scale. If a tool were available to evaluate the distribution and frequency of DMI-resistant populations or genetic groups quickly and globally, a better understanding of *E. necator* evolution under different selection pressures might be gained.

The aim of the present study was to develop a DNA diagnostic tool based on real-time allele-specific PCR using the fluorescent dye SYBR-Green: (i) to identify and quantify the presence of allele-specific mutation linked to a high level of resistance to DMI fungicide; (ii) to identify and quantify both genetic groups A and B; (iii) to obtain data about the distribution of genetic groups and fungicide resistance of *E. necator* in French vineyards. A rapid test would allow more accurate assessment of resistance and epidemic risk with a view to optimising integrated pest management.

2 MATERIALS AND METHODS

2.1 *Erysiphe necator* material and sampling

Powdery mildew isolates ($N = 11$) used to develop the qPCR method came from a laboratory collection (Table 1). Monoconidial isolates were inoculated under sterile conditions on decontaminated grape leaves (cv. Cabernet Sauvignon) in Petri dishes, as described in a previous article.[5] Leaves were placed at the bottom of a Plexiglas settling tower, and conidia were blown in at the top from sporulating leaves (1000–1500 conidia cm^{-2} of leaf). Inoculated leaves were incubated for 12–14 days at 22 °C with a 16:8 h light:dark photoperiod. Fungal material growing on the leaf surfaces was scraped into Eppendorf tubes, dried and stored at −20 °C.

Table 1. Characteristics of *Erysiphe necator* isolates used in this study

Strain[a]	Origin	Genotype	DMI status
TOU 01	Languedoc-Roussillon, France	A	Sensitive
ROU 08	Languedoc-Roussillon, France	A	Sensitive
PVR 43	Languedoc-Roussillon, France	A	Sensitive
PVR 15	Languedoc-Roussillon, France	A	Sensitive
PVR 33	Languedoc-Roussillon, France	A	Sensitive
LAT 12	Aquitaine, France	B	Sensitive
CHL 02	Aquitaine, France	B	Sensitive
GF 10	Aquitaine, France	B	Sensitive
IBA 11	Bangalore, India	B	Resistant
HU3, HU1	Eger, Hungaria	B	Sensitive

[a] All isolates were sensitive to QoI fungicide except isolates HU1 and HU3 which were strobilurin resistant.

Sampling was conducted in French vineyards from May to the beginning of October, with $N = 52$ in 2008 and $N = 59$ in 2009, in six grapevine production regions. Totals of 689 and 720 grapevine lesions were collected over the 2 years. For each sample, 10–15 lesions from leaves (6 mm diameter) were collected and pooled as a sample for each locality. The mycelium of *E. necator* on grape berries was scraped from 15 bunches, pooled in Eppendorf tubes and stored at −20 °C.

2.2 DNA isolation

Total DNA isolated from frozen tissue samples or directly from lesions (infected leaf discs) collected in the vineyards without subculture was extracted with chloroform + isoamyl alcohol, followed by isopropanol precipitation as previously described.[43] After mixing with an equal volume of isopropanol, the DNA pellet was incubated for 2 h at −20 °C, then precipitated by centrifugation for 10 min at 4 °C and 12 000 × *g*. After washing with 70% (v/v) ethanol, the DNA pellet was dried and dissolved in water. The DNA concentration was measured by absorbance at 260 and 280 nm (Genequant Pro; Amersham Bioscience, France). DNA extracts were stored at −20 °C.

2.3 CAPS method

A single nucleotide polymorphism (SNP) created a recognition site of restriction endonuclease *AccI* that allowed the characterisation of A or B isolates by cleaved amplified polymorphic sequence (CAPS) analysis according to the semi-quantitative method previously described.[3,7] Polymerase chain reactions (PCRs) were performed in a 16.5 µL volume containing 1.5 µL of the stock genomic DNA solution diluted 1/3, 1.5 µL of 10× PCR buffer (Eurogentec, France), 1.5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.2 µM of each primer (EN-TUB) (Table 2) and 0.25 U of Taq SilverStar DNA polymerase (Eurogentec). The PCR programme was performed as follows: a first denaturation step of 3 min at 95 °C, followed by 38 cycles of 40 s at 95 °C, 55 s at 58 °C, 55 s at 72 °C and a final elongation step of 5 min at 72 °C. Cleaving reactions were performed in a 10 µL volume with 2 µL of PCR product, 1 µL of 10× buffer No. 4 (BioLabs) and 1.5 U of *AccI* enzyme (BioLabs) at 37 °C for 1.5 h. Restriction fragments were visualised on 2% agarose after staining with ethidium bromide.

Table 2. Primer sequences used in this study

Primers	Sequences (5′–3′) forward	Sequences (5′–3′) reverse	Gene	Genbank accession number	Nt position	Fragment (bp)
qEN	CTTCGGATTTTTGGGTCAGA	GGCAGATCATTGGATTCTT	Cyp51	AF042067	1310–1157	153
qEN 37A	GGGTGGATTTTCATGGTCAC	GCAACTGGCTCAATACATT TAAC**A**	Cyp51	AF042067	88–166	79
qEN 136R	TGGGAAGTTAAAAGATGTCA ACG	TGAGTTTGGAATTTGGACA ATCA**A**	Cyp51	AF042067	418–518	100
qEN 143S	ACCTACTTAAAGCTTTAGAA GTTTCC	TACGGGCAGATGAGCCTAT GCGG	Cyt b			
qEN 143R	ACCTACTTAAAGCTTTAGAA GTTTCC	TACGGGCAGATGAGCCTAT GC**C**	Cyt b			
EN-TUB	GCGAGATCGTAAGCTTGACAC	GGCACGAGGAACGTATTTGT	β-Tubuline	AY074934	38–486	449

2.4 Primers

Primers for the reference *Erysiphe necator* (EN) and specific alleles (for genetic group G37A, and Y136F for DMI resistance) for real-time PCR were designed with Primer 3 software (http://frodo.wi.mit.edu) to be used at the same hybridisation temperature and $MgCl_2$ concentration. Primer pairs were designed in order to detect and quantify different specific alleles (Table 2). They produced allele-specific fragments free of primer dimers or non-specific product contaminants. Two sets of primers consisted of those that were designed to amplify *E. necator* in *CYP51* gene: qEN37A for allele specific to genetic group A, and qEN136R for allele specific to DMI resistance (Table 2). To quantify QoI-resistant allele (G143A), qEN 143S and qEN 143R, two primers previously described by Baudoin *et al.*,[27] were used. To define the optimal annealing temperature and to test the specificity of the primers, PCR gradients were performed in an Eppendorf thermocycler. DNA samples (100–200 ng) were amplified. Twenty-five microlitre PCRs containing 0.1 µM of primers, 1 mM of $MgCl_2$, 0.3 mM of each dNTP and 0.5 U of *Taq*DNA polymerase (Eurogentec, France) were carried out. PCRs were performed using the following amplification parameters: initial preheating at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at variable temperatures (from 55 to 65 °C) for 20 s, extension at 72 °C for 20 s and a final extension at 72 °C for 10 min. The specificity was analysed in a 2% agarose gel in Tris borate EDTA buffer (0.5 M) and by direct sequencing of amplified PCR products (data not shown). They produced allele-specific fragments free of primer dimers or non-specific product contaminants. All primers were tested out on pure isolates that were either 100% genetic group A or 100% group B for the development of q-PCR for the genetic group, and on isolates that were either 100% sensitive or DMI resistant for the development of q-PCR for DMI resistance.

2.5 Real-time PCR protocol

Real-time qPCR reactions were assembled in a 14 µL reaction volume containing 7 µL of Absolute™ QPCR SYBR® Green mix containing thermo-start® DNA polymerase and buffer (Abgene, France), 5 µL of DNA templates and 1 µL of each primer (10 nM), according to the manufacturer's protocol. Each sample was assayed at least in duplicate. PCR reactions were performed in a real-time thermal iCycler iQ (Bio-RAD, Hercules, CA). Three-step PCR amplification was carried out using SYBR® Green fluorescent dye detection under the following conditions: initial preheating at 95 °C for 15 min, followed by 40 cycles at 95 °C for 30 s and at the primer T_m for 1 min (56 °C for genotype primers qEN and qEN37A, 58 °C for DMI primers qEN136 or 60 °C for QoI primers) with a final extension at 72 °C for 20 s. Increase in the fluorescent emission signal from SYBR Green was recorded at the end of the elongation phase at 72 °C for each cycle without signal normalisation. The absence of unwanted products was confirmed by automated melting curve analysis with a temperature increase of 0.5 °C.

2.6 PCR efficiency

To ensure correct normalisation in real-time PCR, amplification efficiencies should be similar. Each PCR system was tested with *E. necator* DNA samples (20 ng μL^{-1}), which were serially diluted in 1 : 10 ratios with distilled water. Amplification efficiencies should be determined from the slope of the log-linear portion of the calibration curve. Specifically, PCR efficiency $= 10^{-1/\text{slope}} - 1$, when the logarithm of the initial template concentration of known DNA amounts (the independent variable) is plotted on the *x*-axis and C_q (the dependent variable) measured by the iCycler™ iQ software (Bio-RAD, Hercules, CA) is plotted on the *y*-axis.

2.7 Real-time quantitative PCR optimisation

Standard calibration curves were constructed with a range of allele-specific frequencies (from 0.36 to 100% of B versus A genetic group, or sensitive versus DMI resistant) using the specific DNAs for the respective alleles. The experiment was performed 3 times and conducted on the three different SNPs. The specific allele was expressed as the relative quantity in relation to an allele common to two genetic groups (EN for *Erysiphe necator*), which was used as an internal calibrator. The difference between the two PCR reactions (ΔC_q) was used to calculate the allele frequency according to the direct correlation between ΔC_q ($\Delta C_q = C_{q\ \text{allele specific}} - C_{q\ \text{EN}}$) and the decimal logarithm of allele-specific frequency. The logarithm of the known allele frequency was plotted as a function of the ΔC_q measured between the two PCR reactions, and a trend line was estimated. To test the linearity of SNP allele quantification, ΔC_q values were used for linear regression analysis. In parallel, a validation method based on determination of the limit of detection (LOD) and the limit of quantification (LOQ) was used in order to avoid the occurrence of false positives.[34] By assuming the normal distribution of measured ΔC_q values, LOD and LOQ were calculated from the residual standard deviation of the linear regression data according to LOD $= 3 \times (S_{xy}/b)$ and LOQ $= 10 \times (S_{xy}/b)$, where S_{xy} is the residual standard deviation and *b* is the slope of the linear regression equation.

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In a second assay, the same concentrations of *E. necator* DNA (10 ng μL^{-1}) were serially diluted (1 : 1, 1 : 10, 1 : 10^2, 1 : 10^3) in *Vitis vinifera* DNA (60 ng μL^{-1}) to investigate any possible influence of host DNA on amplification of pathogenic DNA. The experiment was performed twice. Foliar discs contaminated with each genotype (i.e. A or B) were used to test a range of mixes, e.g. three foliar discs of A genotype and seven foliar discs of B genotype, giving 30% of genotype A in the mixed sample. DNAs were isolated as previously described. The ΔC_q values of samples taken as unknown were then used to calculate the allele frequency according to the trend line equation obtained previously. The correlation between known and measured allele-specific frequencies obtained for each SNP was established.

3 RESULTS

3.1 Establishment of a real-time PCR system for SNP quantification

PCR efficiencies were similar for the EN reference, 37A and 136R specific allele PCR assays, at 1.23, 1.22 and 0.99 respectively, and they were derived from the slope of the standard curves (Fig. 1). The C_q (quantification cycle) values ranged from 19 to 32 for reference *E. necator* primers (EN) (Fig. 1A), from 21 to 30 for genetic group A allele-specific primers (37A) (Fig. 1B) and from 23 to 38 for DMI-resistant allele-specific primers (136R) (Fig. 1C). Values over 32, 30 and 38, respectively, for EN, 37A and 136R were considered as negative (false positive). The C_q values corresponded to a detection limit of 2 pg of *E. necator* DNA, 0.06 ng of genetic group A DNA and 1.4 ng of DMI-resistant DNA according to specific calibration curves for DNA samples (Fig. 1).

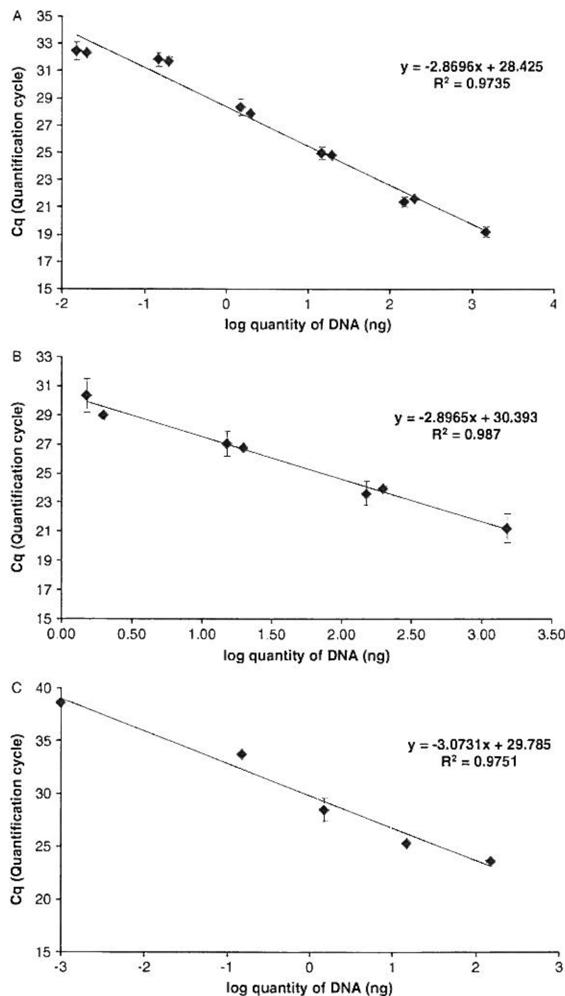

Figure 1. PCR efficiencies by plotting the quantification cycle (C_q) versus the amount of log quantity of template DNA (ng) for *Erysiphe necator*. A: efficiency for *E. necator* specific allele (q-EN); B: efficiency for group A specific allele (q-EN-37A); C: efficiency for DMI-resistant allele (q-EB-136R). PCR efficiency $= 10^{-1/\text{slope}} - 1$.

3.2 LOD and LOQ values and validation

Regression coefficients of calibration curves (Fig. 2) were 0.983 and 0.986, and residual standard deviations S_{xy} were 0.679 and 0.890 for group A allele-specific 37A (Fig. 2A) and DMI-resistant allele-specific 136R (Fig. 2B) primer sets respectively. High correlations were found between allele-specific frequencies and ΔC_q values. The absolute LOD values calculated from residual standard deviations of the linear regression data of 37A and 136R alleles were 0.72 and 0.85% respectively, and the LOQ values were 2.40 and 2.85% for 37A and 136R alleles respectively. In addition, the LOD and LOQ values of QoIs for the 143R allele were 1.04 and 3.49% respectively (data not shown).

The frequencies measured on pooled DNA samples were also compared with 'known' frequencies and were well correlated for group A and DMI-resistant specific alleles, with $R^2 = 0.967$ and 0.990 respectively (data not shown). Finally, allele frequencies from the pooled samples were determined by qPCR and compared with those obtained by the CAPS method. On agarose gel, the

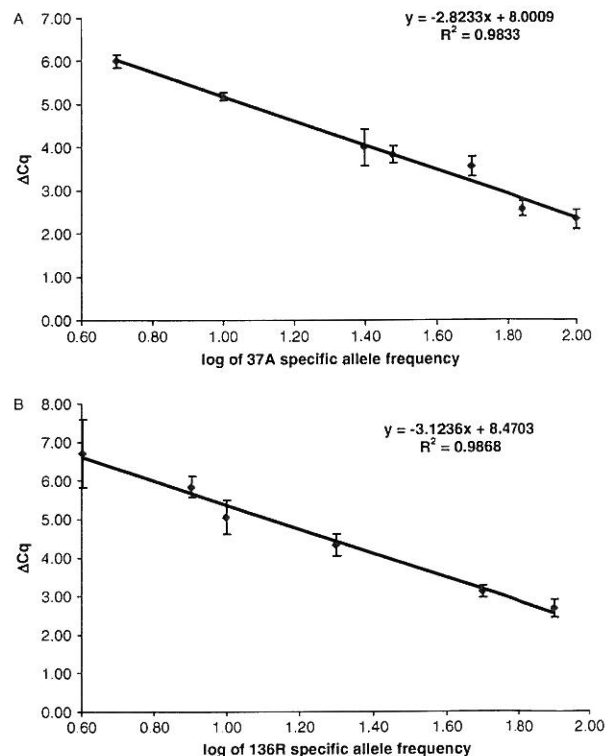

Figure 2. Linear relationship between the frequency of genetic group A allele qEN-37A (A) or DMI-resistant allele qEN-136 (B) in *Erysiphe necator* and ΔC_q values determined with real-time PCR assays.

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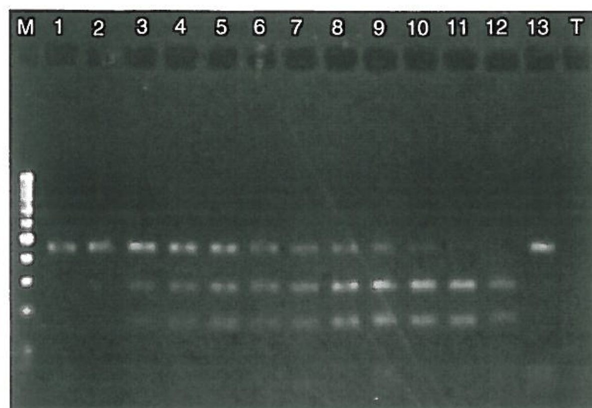

Figure 3. Cleaved PCR fragments obtained from 13 DNA samples containing different ratios of genetic groups A and B. Lane M contains molecular marker (100 bp ladder); lanes 1 and 13: 100% of genetic group A showing a unique fragment of 449 bp; lanes 2 to 10: different ratios of group A (90, 80, 70, 60, 50, 40, 30, 20 and 10%) mixed with group B; lanes 11 and 12: 100% of genetic group B; lane T contains a water control (no DNA).

Table 3. Distribution of genetic group A specific allele and DMI-resistant allele of *E. necator* in various French vineyard regions in 2008

Region	*N*	Mean percentage of G37A allele (group A) (± SEM)	Mean percentage of Y136F allele (DMI resistant) (± SEM)
Aquitaine	6	0(±0)	0(±0)
Burgundy	7	0.8(±0.80)	5.51(±3.49)
Champagne-Ardennes	6	0.96(±0.96)	0(±0)
Languedoc-Roussillon-1	6	11.06(±7.27)	0(±0)
Languedoc-Roussillon-2	10	75.13(±11.84)	1.43(±1)
Midi- Pyrénées-1	10	10.17(±6.86)	0(±0)
Midi-Pyrénées-2[a]	5	1.16(±1.16)	18.77(±18.82)
PACA	2	9.39(±6.25)	2.62(±2.62)
National average	52	18.37(±4.73)	2.92(±1.87)

[a] Only Midi-Pyrénées-2 region showed QoI-resistant alleles with a mean of 5.16 ± 5.67%. The national average was 0.6 ± 0.6%.

presence of a specific band (449 pb) of group A was detected in mixtures containing from more than 10% to 100%, and conversely for the group B. The different proportions of the two alleles were observed in mixtures but could not be estimated exactly (Fig. 3). In parallel, these same mixtures considered as unknown samples were tested with the present qPCR method. The values of frequencies measured were totally correlated with the theoretical values, with a correlation coefficient of 0.977 (data not shown). Comparison of both methods by analysis of field samples, either of pooled samples (q-PCR) or of an average of 10 individual samples (CAPS), showed no significant difference ($P = 0.925$) for percentages of allele above 29% (data not shown).

3.3 Frequency of group A allele in field populations of *E. necator*

To assess the importance of genetic group A distribution in French vineyards, 52 and 59 field samples were typed in 2008 and 2009 in this study (Tables 3 and 4). In 2008, 18.37% of field samples were quantified as genetic group A, but only 6.60% in 2009 (Tables 3 and 4). Nationwide, for the total sampling carried out in 2008 and 2009, a mean of 12.14% of these samples was group A and a mean of 87.89% was group B (Table 5). The frequencies of group A ranged from 0 to 100%, with variability depending on the samples and on vineyard areas (Tables 3 to 5). In 2008 only the samples from area LR2 ($P < 0.0001$) and in 2009 those from area LR1 ($P < 0.02$) were significantly different from those of other areas, with 75.13 and 24.08% of group A present (Tables 3 and 4, Fig. 4A). The variability observed in LR areas in 2008 and 2009 is related to the earliness of the samplings ($P < 0.0001$) and the organ considered. This was reinforced by the entirety of samples obtained from leaves, where a significantly higher frequency of group A (17.35%, $P = 0.028$) than that collected on grape berries (5.47%) was found (Table 5).

With regard to the samples from Mediterranean vineyards in 2008 and 2009, such as the Languedoc-Roussillon (LR1, LR2) and PACA (PA) regions, it was remarked that these regions exhibited a significantly higher frequency of group A than other areas, with an average of 37.08%, and only 2.44% in other regions ($P < 0.0001$) (Table 5, Fig. 4A).

Table 4. Distribution of genetic group A specific allele and DMI-resistant allele of *Erysiphe necator* in various French vineyard regions in 2009

Region	*N*	Mean percentage of G37A allele (group A) (± SEM)	Mean percentage of Y136F allele (DMI resistant) (± SEM)
Aquitaine	2	0(±0)	12.26(±12.30)
Burgundy	13	1.53(±0.63)	1.06(±0.95)
Champagne-Ardennes	3	4.21(±4.21)	37.27(±31.44)
Languedoc-Roussillon-1	13	24.08(±10.93)	11.77(±7.63)
Midi-Pyrénées-2[a]	24	0.35(±0.26)	33.40(±8.44)
Rhône-Alpes	4	8.89(±8.86)	13.67(±11.13)
National average	59	6.60(±2.71)	19.65(±4.49)

[a] Only the Midi-Pyrénées region exhibited QoI-resistant allele with a mean of 23.18 ± 8.0%. The national average was 9.43 ± 3.61%.

3.4 Frequency of DMI-resistant allele and QoI-resistant allele in the field population of *Erysiphe necator*

A total of 111 samples collected in 2008 and 2009 were used to assess the presence of specific allele involved in DMI resistance (Y136F) (Fig. 4B) and in QoI resistance (G143A) (Fig. 4C). In 2008, out of 52 samples assessed, only ten showed the presence of Y136F DMI-resistant allele in French vineyards, and the allele was significantly quantified in seven samples, with a mean percentage of 2.92% (Table 3). Only two samples, from the Burgundy and Midi-Pyrénées areas, exhibited a high frequency of DMI-resistant allele: 25.07 and 93.85% respectively. In 2009, among 59 samples, the Y136F allele was detected in 32 samples, with a mean percentage of 19.65% in all areas (Table 4), with a marked presence in the Midi-Pyrénées (MP2), where nine samples with more than 50% of DMI-resistant allele were found. During the 2 years, the mean percentage of Y136F allele found in different regions increased from 2.92% in 2008 to reach 19.65% in 2009. Nationwide for the 2 years, 11.81% of samples had the DMI-resistant allele, and 88.19% had the sensitive allele at position 136, with unequal distribution into various regions (Table 5, Fig. 4B). A significant difference was found in the regions at a threshold of 5% ($P = 0.006$), but not in

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Table 5. Mean distribution of specific alleles for DMI resistance, QoI resistance and genetic group A of *Erysiphe necator* in French vineyard regions in 2008–2009

Region	*N*	Mean percentage of G37A allele (group A) (± SEM)	Mean percentage of Y136F allele (DMI resistant) (± SEM)	Mean percentage of G143A allele (QoI resistant) (± SEM)
Aquitaine	8	0(±0)	3.07(±3.05)	0(±0)
Burgundy	20	1.53(±0.48)	1.06(±1.36)	0(±0)
Champagne-Ardennes	9	2.04(±1.47)	12.42(±10.99)	0(±0)
Languedoc-Roussilon-1	19	19.47(±8.03)	8.06(±5.45)	0(±0)
Languedoc-Roussillon-2	10	75.13(±11.85)	1.43(±1.00)	0(±0)
Midi-Pyrénées-1	10	10.17(±6.86)	0(±0)	0(±0)
Midi-Pyrénées-2	29	0.49(±0.28)	30.88(±7.63)	20.25(±6.79)
PACA	2	9.39(±6.25)	2.62(±2.62)	0(±0)
Rhône-Alpes	4	8.86(±8.86)	13.67(±11.13)	0(±0)
National average	111	12.11(±2.69)	11.81(±2.65)	5.29(±1.98)
Mediterranean vineyards (LR, PA)	31	37.08(±2.69)	5.57(±3.28)	0(±0)
Outside Mediterranean vineyards (A, B, CA, RA, MP)	80	2.44(±1.01)	14.24(±3.42)	12.50(±3.42)
Leaf samples	62	17.35(±4.45)	13.48(±4.04)	9.05(±3.16)
Grape berries samples	49	5.47(±2.05)	4.24(±2.73)	15.28(±4.48)

the Mediterranean area ($P = 0.143$) or in the organs, i.e. leaves or berries ($P = 0.245$), and no sample belonging to 100% group A exhibited the DMI-resistant allele.

Allele-specific G143A qPCRs were performed as described by Baudoin *et al.*[27] and enabled the presence of QoI resistance in French vineyards to be detected for the first time in 2008. Only one sample from the MP2 area, which is already highly resistant to DMI (93.85%), exhibited QoI-resistant allele (30.99%), leading to 0.6% of QoI-resistant allele in 2008 at the nationwide level (Table 3). In 2009, the G143A allele involved in QoI fungicide resistance was detected in 11 samples with a mean of 9.43% (Table 4), all coming from the Midi-Pyrénées region (MP2) as in 2008. It was noticed, as in 2008, that samples exhibiting QoI-resistant allele frequently showed DMI-resistant allele (8/11). Overall, nationwide for the 2 years, the mean percentage of QoI-resistant allele detected reached 5.29% (Table 5, Fig. 4C).

4 DISCUSSION AND CONCLUSION

The primers developed here were able to amplify DNA of *E. necator* and to identify genetic group A and DMI-resistant alleles. With qPCR assays it is thus possible to detect and quantify group A and DMI-resistant populations under the 10% detection limit of the CAPS method, with quantification limits of 2.40 and 2.85% and detection limits of 0.72 and 0.85% respectively. Several genotype quantifications can be specified on the same sample or population, such as genetic group A or B, DMI resistance and QoI resistance frequencies.

These quantitative methods could be used for dynamic population studies to measure the genetic group evolution of *E. necator* throughout the growing season, and to monitor resistance to DMI and QoI fungicides in the vineyard. In the case of an obligate parasite such as *E. necator*, being able to identify and quantify pooled samples represents a real step forward for monitoring vineyard populations of the pathogen.

4.1 A/B ratios and geographic distribution

For genetic group determination, a qualitative test exists for screening field population by nested PCR or CAPS methods,[3–14,16,22] but quantitative tests need many samples with individual genotype determination. The method developed here was initially designed as an alternative to traditional quantitative and qualitative tests, and qPCR analysis of group A and B mixtures showed a better sensitivity than the CAPS method. The A/B ratios observed in the 111 field samples were variable, with an average of 12.11% of group A and 87.89% of group B (Table 5, Fig. 4A). Thus, in French vineyards, group B is predominant, with its proportion varying according to region, organ (leaves/berries) and time of collection.

It was noted that there were important variations in allele frequencies within a region and between regions. Concerning the allele of group A, part of the variability is due to the date of collection and to the organ harvested. Indeed, samples collected at the beginning of the season are significantly richer in group A than in populations collected later ($P < 0.0001$), in particular in vineyards where group A is present (LR, PACA, RA, MP1). This is also related to the ecological requirements of genotype A, which appears early, at the beginning of the growing season. For example, in 2008, in the Languedoc-Roussillon-2 area, samples harvested early were significantly different from those from other areas ($P = 0.0001$), with 75.13% in group A. On the other hand, the samples from the Languedoc-Roussillon-1 area in 2008, collected 2 months later, exhibited only 11.06% in group A. Although the samples did not come from the same plot, as described in a previous article, group A isolates appear to be prevalent more at the beginning of the growing season and then tend to decrease in the vineyard as the season moves on, to the benefit of group B,[8,11,13] before preparing to overwinter in dormant buds of the grapevine. Globally, group A was prevalent more on leaves (17.35%) than on grape berries (5.47%) (Table 5), suggesting that group A isolates are less aggressive on berries than group B isolates, as reported for other genetic groups of fungus such as *Botrytis cinerea* Pers.[44] This result is in agreement with previous studies describing group A as less aggressive than group B,[13,14] or group A as less prevalent in grape berries than group B.[11,12] This reinforces the hypothesis that the two groups could have different ecological requirements,[7] and that the fungicide pressure exerted on group A was less important than on genetic group B which was present throughout the growing season. In previous studies,

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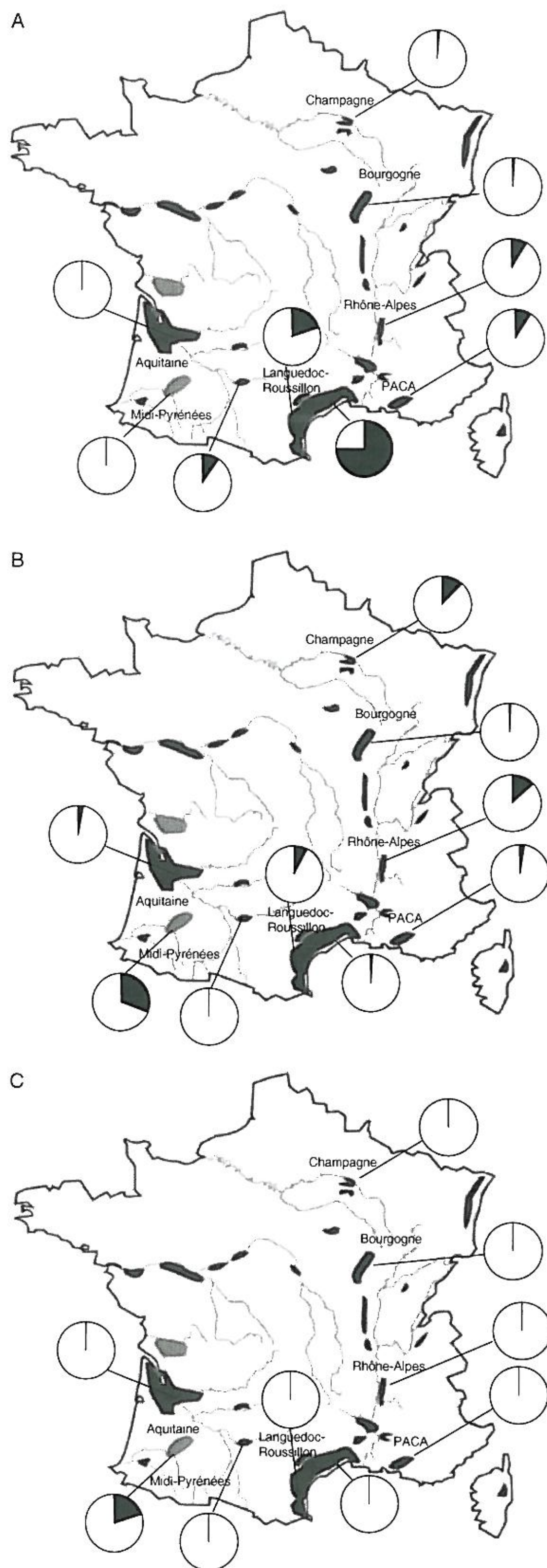

Figure 4. Comprehensive mean distribution of specific alleles of *Erysiphe necator* genetic group A (A), DMI resistant (B) and QoI resistant (C), in different vineyard regions.

group A seemed to be prevalent more in Mediterranean areas than in other areas.[1,8,13] The qPCR assays here showed that the areas bordering the Mediterranean Sea exhibit a significantly higher frequency of group A (37.08%) than other areas (2.44%, Table 5). This suggests the possibility of a geographical variation in the population structure of *E. necator* owing to climatic factors and/or the sensitivity of local cultivars to grapevine powdery mildew.

4.2 Fungicide-resistant allele distribution

Determination of fungicide sensitivity in pathogen population and which genetic group is involved is the first important step in management of fungicide resistance. The most commonly used test for determining DMI sensitivity of *E. necator* isolates was a biological test involving mycelium growth inhibition. This required biological tests to be conducted at one or several concentrations and was very fastidious, in particular with an obligate parasite such as *E. necator*. After this came the nested PCR method, used to detect DMI resistance based on Y136F, but it was not quantitative.[39] The present method was therefore designed to be able to quantify the proportion of a resistant allele in a population, with the possibility of pooling samples. The resistant allele Y136F exhibited a national average of 11.81%, which strengthens the idea that the DMI resistance of grapevine powdery mildew in French vineyards is limited. However, the status can be very contrasted, with some plots showing 100% of DMI-resistant allele and others 0%. The present authors would therefore suggest that the variability observed with resistant alleles 136F and 143A could essentially be the result of the strength of the epidemic, and genetic variability, as well as the choice of fungicide treatment and cultivation practices. Thus, plots where vines are close together can present very different frequencies of the specific alleles. In addition, sexual reproduction may also play an important part in the spread of resistant alleles, depending on the sexual capacity of the genetic group.

DMI resistance was not strictly monoallelic, and various studies have shown the polygenic aspect of this fungicide resistance, involving: (i) mutation of the DMI target enzyme 14α-demethylase CYP51;[23,25,26,45] (ii) overexpression of the *CYP51* gene;[45–50] (iii) overexpression of ATP binding cassette (ABC) transporters coding for efflux pumps;[51,52] (iv) unknown mechanisms conferring weak resistance.[8,12,53,54] However, for an obligate pathogen such as powdery mildew, the major mutation in position 136 or its equivalent has been described,[23,25,26] even if other minor mechanisms may coexist.[12,22] The qPCR assay can be used for detection and quantification of resistant Y136F allele in *B. graminis*, another powdery mildew.[32] A method to determine the development of fungicide resistance in grapevine powdery mildew populations is a basic step in being able to predict possible trends in fungicide resistance on a large scale. The qPCR assay described in this study is applicable for high-throughput detection of the resistant Y136F allele in more than 100 populations. As cross-resistance is widespread between triadimenol, triadimefon, propiconazole, tebuconazole and cyproconazole[12–22] in MP2 populations, where the frequency of 136Y increased from 18.77% in 2008 to 33.40% in 2009, the authors would suggest that these fungicides cannot control the disease everywhere, in particular in six plots where the resistant allele was present at more than 90%. In this event, a fungicide with a mode of action other than sterol demethylase inhibitor would be required. More alarmingly, in some populations in the Midi-Pyrénées (MP2), resistance was also found to a QoI fungicide, which acts on cytochrome b, a mitochondrial respiration target,[55] combined with DMI-resistant

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Table 6. Mean distribution of alleles specific to DMI and QoI resistance, depending on the percentage of group A of *Erysiphe necator*

	Percentage (± SEM)[a]	
Group A classes	DMI allele	QoI allele
0–15%	37.72(±7.28) a	41.93(±12.61) a
15–85%	15.34(±8.42) b	0(±0) b
85–100%	0(±0) c	0(±0) b

[a] a, b, c: significantly different at a threshold of 0.05%.

allele. This suggests that fungicides may be selecting double resistance in *E. necator* populations. This is the first report on double fungicide resistance in grapevine powdery mildew and, following the development of double resistance in populations of *E. necator*, should be of interest for controlling epidemics and the spread of resistant alleles in relation to nuclear or mitochondrial heritability. The different tools reported here would be very beneficial for *E. necator* management. It would be interesting to monitor the evolution of the sensitivity of the Midi-Pyrenées-2 plot for several years and to modify the treatment programme, which has systematically consisted in four DMI and two QoI treatments per growing season. The monitoring carried out in 2009 corroborates the double resistance found in 2008, with seven detections of the two resistant alleles (Y136F and G143A).

The possibility of combining quantification of resistant alleles with the presence of genetic group A or B could throw light on the role played by the genetic group in the development of resistance in relation to the biological characteristics of the two groups, and could also improve pest management. Indeed, genetic group A, which is more prevalent in Mediterranean vineyards, might winter in the dormant buds but without sexual reproduction in France.[12,14–16] Moreover, group A isolates were significantly more sensitive to the different fungicides than group B isolates in the absence of specific DMI-resistant allele or QoI-resistant allele.[8,12] Indeed, the present authors have previously shown that the sensitivity baseline of monoconidial isolates to DMIs, quinoxyfen and azoxystrobin was significantly different in the two genetic groups A and B, with differential factors between groups A and B of 15.6, 4.7 and 3.5 for triadimenol, penconazole and tebuconazole DMI fungicides. The study carried out on isolates of group A or B having never been handled with quinoxyfen and QoI fungicides also showed a difference of a factor 2 between the two groups, with the group B less sensitive than the group A. The results of the present study support previous work on the difference in fungicide sensitivity of the two genetic groups A and B. Indeed, populations exhibiting alleles resistant to fungicides belonged in the majority to group B. Thus, populations exhibiting less than 15% of group A had the strongest mean percentage of resistance (37.72%), and those containing 15–85% or 100% of group A showed only 15 or 0% of resistant allele respectively (Table 6). As regards QoI resistance, only populations exhibiting 100% of group B showed QoI resistance, with a mean of 41.93%. These results reinforced the role of group B in the resistance of fungicides in populations of *E necator*.

This new diagnostic method has numerous advantages: (i) the method is not based on expensive fluorescent labelled primers or probes; (ii) it is a tried and tested assay that requires no post-PCR processing (RFLP or nested PCR); (iii) it promises to be time saving and precise enough to allow detection of weak genetic associations; (iv) it can serve to estimate genetic variability on a large scale owing to the possibility of working with pooled samples versus individual symptoms. Moreover, this is the first study to offer a wide overview of *E. necator* diversity in French vineyards more easily than nested PCR or CAPS on individual samples. From now on, it will be possible to specify the distribution of the various genetic groups and to test different hypotheses. Furthermore, early detection of resistant alleles at low freqency, coupled with risk evaluation, would allow implementation of antiresistance strategies in order to prolong the cost effectiveness and lifetimes of fungicides. This q-PCR assay can help wine growers to manage fungicide treatments and reduce the risk of appearance of resistance and its spread. Indeed, knowing which genetic groups are present at the beginning of the season (A and/or B) will allow them to adjust the use of QoI or DMI fungicides. An assessment of the sensitivity of a vineyard plot in year N will mean anticipation for year $N + 1$ and the choice of a more appropriate treatment programme. In addition, in France, for example, out of 30 approved chemical compounds, 14 contain a DMI and six contain a QoI fungicide. There is not much leeway, and proper management of the risk of resistance becomes essential. The frequencies of the different specific alleles could be used to integrate pest management in vineyards and to obtain a better understanding of the role of groups A and B. This would help in obtaining a more accurate assessment of exposure to genetic variability in vineyards nationwide and throughout the growing season. In particular, knowledge of how long it takes for populations to move from being sensitive to becoming resistant with loss of fungicide efficacy is important for well-directed fungicide resistance management programmes. The assay could prove useful in the routine monitoring of pest management in vineyards, which would in turn provide timely warning for wine growers.

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