Chapter 16
Monitoring Resistance in Obligate Pathogens by Bioassays Relating to Field Use: Grapevine Powdery and Downy Mildews

Marie-France Corio-Costet

Abstract Monitoring is required to detect and estimate the sensitivity status of pathogen populations to fungicides for the effective control of diseases in a setting of sustainable disease management. Several biological methods are available to detect and to quantify the emergence and evolution of fungicide resistance in obligate parasites in the vineyard such as *Erysiphe necator* and *Plasmopara viticola*. To perform good monitoring, particular attention must be paid to the sampling in the vineyard that will serve to determine baseline and discriminant doses. This chapter first describes the methods used to study fungicide resistance in the field and then compares monitoring results for various fungicides and the role of pathogen diversity. This information will help users to choose the most suitable method and to manage resistance.

Keywords CAA • DMI • *Erysiphe necator* • Fungicide • Grapevine • Monitoring resistance • *Plasmopara viticola* • QoI

Abbreviations

CAA	Carboxylic acid amid
A.i.	Active ingredient
DD	Discriminant dose
DMI	C14-sterol demethylase inhibitor
EC_{50}	Effective concentration of fungicide inhibiting pathogen development at 50 %
MIC	Minimum inhibitory concentration
QiI	Quinone inside inhibitor
QoI	Quinone outside inhibitor

M.-F. Corio-Costet (✉)
INRA, UMR Santé et Agroécologie du vignoble (UMR 1065, SAVE), ISVV, LabexCOTE, Rue Edouard Bourlaux, CS 20032, 33882 Villenave d'Ornon Cédex, France
e-mail: coriocos@bordeaux.inra.fr

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q-PCR Quantitative polymerase chain reaction
RF Resistance factor

16.1 Introduction

Since their arrival on the European continent, the obligate parasites downy (*Plasmopara viticola*) and powdery mildew (*Erysiphe necator*) have caused much damage in European vineyards. In 1952, only 7 years after the appearance of *E. necator* on the European continent, 50–70 % of the grapevine harvest in French vineyards had been destroyed by powdery mildew (Galet 1977). Similarly, the invasion of *P. viticola* in 1878 led to considerable loss of yield, which in 1882 was total in some French vineyards (Lafon and Bulit 1981). Since then, research has led to the development of phytosanitary protection products such as copper, sulfur, and other multisite fungicides, which are still used but sometimes have limited efficacy depending on the climate and epidemic pressure. Numerous fungicides have been patented during the last 60 years. Today, for example, 60 commercial products have been approved in France to control downy mildew and 31 for powdery mildew, but only 9 different modes of action are available (Baudet and Lejeune 2014). However, while these products have a good bioavailability and are often used in low doses, their specificity in terms of mode of action, which is rather uni-site, drives the pathogen population to adapt. The result is the occurrence of resistance, which is characterized by the appearance individuals that are less sensitive and then by resistant populations that can lead eventually to a loss of efficacy in the field.

The term “resistance monitoring” is used to observe, detect, and monitor changes in the sensitivity of a population of targeted pathogens in the field. This involves continuous surveillance over several years and involves many locations under different epidemic pressures. Well-carried-out monitoring is the cornerstone of good resistance management. Fungicide monitoring is usually performed at the national or regional level, but also by associations such as the FRAC (Fungicide Resistance Action Committee). All partners organize monitoring and pool their data, leading to issuing recommendations for the use of different fungicides (Brent and Hollomon 2007a, b).

How are the initial events of resistance emergence discovered, and how can its dispersal, increase, and persistence be prevented (Fig. 16.1). By identifying the resistance risks of fungicides, all the partners determine sensitivity baselines and discriminant doses for the fungicide, which allows a monitoring strategy to be set up, the epidemic to be monitored nationwide, and the surveillance recommendation to be issued, e.g., via the French national memorandum on mildews.

Furthermore, depending on the mode of resistance acquisition, be it monogenic or multigenic, and according to the characteristics of the pathogen, whether it is haploid or diploid (case of recessive resistance), the follow-up methods can vary. Indeed, in the case of a disruptive resistance which appears very suddenly and the presence of an

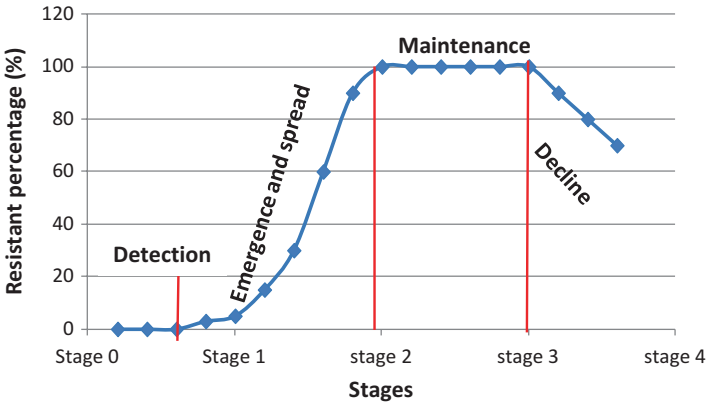

Fig. 16.1 Theoretical evolution of resistance phenomenon with different stages

allele (e.g., allele G143A involved in QoI resistance) confers strong resistance straightaway, a discriminant dose is quite easy to determine (e.g., 100–1000 fold the dose of the MIC for sensitive isolates, Chen et al. 2007; Corio-Costet et al. 2011; Gisi et al. 2002; Grasso et al. 2006). If the gene involved in the resistance is known, it is then quite easy to work out molecular tools (Sirven and Beffa 2003; Baudoin et al. 2008; Dufour et al. 2011). On the contrary, in the event of multigenic or progressive resistance, several years are necessary to observe the appearance of resistance in the field. This is the case for DMI resistance of grapevine powdery mildew (Scheinpflug 1994; Délye and Corio-Costet 1994; Steva and Clerjeau 1990) and the CAA resistance of downy mildew, which is monogenic, but recessive (Gisi et al. 2007; Blum et al. 2010a). In these cases, it is more difficult to determine a discriminant dose, if one wishes to identify the first cases of a decrease of sensitivity and to pay attention to possible false positives, because sympatric populations may sometimes be present (e.g., *E. necator*) (Corio-Costet 2007; Corio-Costet et al. 2003). Often, no naturally occurring resistant isolates are obvious during the first years of viticultural use of these fungicides.

A visible resistance problem is often difficult to predict, and what happens is that monitoring tends to follow the indications for loss of control. Results are then obtained too late to allow any action other than withdrawal of the product. However, caution is needed concerning the use of the term "resistance." More or less resistant isolates can be identified but with low frequency, so this does not systematically lead to a loss of efficacy in the vineyard. Conversely, efficacy losses do not always translate into resistance, because inadequate cultural practices might be involved such as under dosage and poorly, directed, or erroneous treatments. Often, the detected resistant populations reach frequencies superior to 0.01 (1 %) (Fig. 16.1). On the other hand, if monitoring preceded any major decrease in performance and avoidance strategies were either already in operation or being introduced, then the degree of success will vary. Early monitoring from the beginning of the use of a new

Table 16.1 Phases of monitoring and resistance management

Timing	Resistance monitoring activities	Value
Before the beginning of use in the vineyard	Sampling, baseline sensitivity, and distribution of populations	Knowledge of resistance risk and strategy of use
During years of use	Monitoring in different areas (different cultural practices, climates, varieties, etc.)	Practical performance and strategy for use
Case of suspected resistance	Intensification of monitoring and targeted research	Resistance is confirmed or not, study of cross resistance and resistance fitness
Trial of erosion	Plots where fungicide was used at least three times per year	Potential weakening of fungicide and knowledge on resistance selection speed
Subsequently	Monitoring for spread or decline of resistance	Performance and new strategies
In the future used with apparent stability or decline in resistance	Monitoring more spaced out in time and followed by resistance	Value of fungicide in mixture and/or if it is used against other diseases in the same vineyard

Modified from Brent (1994)

molecule makes it easier to track the onset of resistance and its evolution over time. Spot guidance can be obtained and given to growers if the monitoring is rapidly set up, hence the interest of developing molecular methods (e.g., q-PCR), which are easy to develop when the resistance is monogenic and the gene coding for the target is known (Table 16.1).

Below, I describe how to detect resistance during monitoring and what can be done (1) to better detect the emergence of resistance, (2) to develop tools or mapping the risk of resistance, and (3) to assess the dispersal of alleles involved in resistance. Three examples concerning resistance to sterol-C14-demethylase inhibitors (DMIs), quinone outside inhibitors (QoIs), and carboxylic acid amide (CAA) are given to explain the monitoring that can be done in the vineyard with obligate pathogens.

16.2 How Can One Detect Fungicide Resistance in Grapevine Mildews?

Two obligate pathogens, an ascomycete fungus ectoparasite (*E. necator*) and a chromites, oomycete endoparasite (*P. viticola*), are treated very differently in the vineyard: the former with single products and the latter with mixtures of fungicides (2, 3, and nowadays 4).

Firstly, it is essential to determine the variation in sensitivity to a fungicide within the target population. Great care must be taken in extrapolating results of laboratory

tests, where one can use isolates or populations that have lost their aggressiveness or doses controlling growth that do not correspond to those used in the field. The latter point requires taking into account the quantity of active ingredient used in the field, and especially the spray volume, because this will have a direct impact on the efficacy of the treatment. This is why field samples should be collected to test the sensitivity levels before the fungicide is used in order to provide baseline data for subsequent comparisons and determine the first discriminant doses.

16.2.1 Sampling

There are two methods of sampling: either extensively in order to create pools and work on a maximum number of geographical sites or by targeting vineyards, where the pressure of disease is usually strong (e.g., vines with flag-shoot symptoms for *E. necator*, environment leading to disease, or vineyards with gobelet pruning, wet areas for *P. viticola*) and subjected to more treatments than other sites where cultural practices restrict mildew spread. The two approaches are complimentary, one giving an overall view of the real status of resistance and the second making it possible to detect and anticipate the spread of resistance.

The quality of the sample plays a big part in monitoring success. Upon arrival in the laboratory, samples with *E. necator* or *P. viticola* are examined with a dissecting microscope to determine the presence of conidia or sporangia. If the leaves or grapes are sporulating, inoculum can be used directly for subculturing or bioassays, or the leaves and grapes are placed in a moist chamber to reinitiate sporulation. However, it is always possible to subculture inoculum even from old leaves infected with E. *necator* and even those harvested in October in Europe when there are cleistothecia (Délye and Corio-Costet 1998; Cartolaro and Steva 1990; Gadoury and Pearson 1991; Evans et al. 1996; Miazzi et al. 1997). For downy mildew, it is possible to recover sporangia after incubation of symptoms in a wet chamber and then to perform a bioassay (Clerjeau et al. 1985; Genet et al. 1997).

Samples are collected during the growing season in different vineyards treated or not treated with fungicide. Usually for a random test, 10–50 leaves exhibiting lesions are collected from each plot. Collecting can be arbitrary at several points in a vineyard. The leaves are cryopreserved in an icebox or packed with other leaves and put in newspaper before rapid postal dispatch. Most samplings are performed in August or September after the final spraying has taken place. However, sampling at the beginning of the season can be very useful for knowing the extent of selection pressure during the previous growing season and for having data about the fitness of resistant isolates.

A sufficient number is needed because single conidia or monosporangial isolates can then be used to build reference collections (Délye et al. 1997b; Erickson and Wilcox 1997; Sierotzki et al. 2005; Toffolatti et al. 2007).

16.2.2 Multiplication and Collection of Isolates

16.2.2.1 Grapevine Powdery Mildew (*E. necator*)

E. necator, which is a strict parasite, requires grapevine leaves of good quality as nutritional support. Susceptible grapevine cultivars are used for experiments (e.g., Cinsaut, Cabernet Sauvignon, Chardonnay, Blauberger, etc.). To perform calibrated bioassays, young cuttings (6–8 weeks) are used. The more susceptible polished leaves are used for inoculum multiplication and bioassays (Debieu et al. 1995; Erickson and Wilcox 1997; Evans et al. 1996; Miazzi et al. 1997; Cartolaro and Steva 1990; Gadoury and Pearson 1991; Colcol et al. 2012). A technique with *E. necator* isolates is to culture them on leaves or on leaf disks (9–20 mm diameter) either surface sterilized for 10 min by immersion in calcium hypochlorite solution (50 g l^{-1}) or disinfected in 10 % bleach with 0.1 % Tween 20 for 2 min, or with 50 % ethanol for 30s, then rinsed in sterile water and blotted dry between paper towels, and kept alive on water agar (20 mg l^{-1} and 30 mg.l^{-1} of benzimidazole or 15 g l^{-1} agar) (Délye et al. 1997b; Erickson and Wilcox 1997). Other authors inoculate *E. necator* conidia under sterile conditions onto the upper surface (adaxial side) of grape leaves in a Plexiglas spore-settling tower (Aslam and Schwarzbach 1980; Reifschneider and Boiteux 1988; Debieu et al. 1995), by blowing 700–1000 conidia per cm^2 of leaf (counting with Malassez cell or Coulter counter) with an air pump connected to a flexible plastic tube terminating in a Pasteur pipette. The inoculated leaves in Petri dishes are removed from the tower and the lids replaced. They are then allowed to grow for 12 days in a growth chamber at 22 °C with 16 h day^{-1} light. Afterward, the inoculum can be used for different tests.

To create an isolate collection, monoconidial isolates can be obtained by picking a single powdery mildew conidium from mildewed samples within a laminar flow hood, after observation with a dissecting microscope, using an eyelash or a camel hair fastened to a holder or a platinum thread. Infections are established on 18 mm diameter leaf disks excised from leaves decontaminated as described above. Between each picking, the eyelash or the thread is disinfected by immersion in ethanol (70%, v/v). Afterward, the inoculated disks or leaves are placed in a growth chamber at 22 °C for 11–14 days (Corio-Costet 2007; Debieu et al. 1995; Erickson and Wilcox 1997).

16.2.2.2 Grapevine Downy Mildew (*P. viticola*)

As for the experiment with *E. necator*, grapevine leaves should be available from cuttings in a greenhouse. Lesion-bearing leaves are cut, moistened, and incubated in a plastic bag overnight at 20–22 °C to promote sporangial growth. Leaves with lesions are usually washed with water to remove any fungicide residue, or freshly produced sporangia may be harvested with a paintbrush and dispersed in water at 4 °C (Bissbort and Schlösser 1991). Sporangial populations are subcultured one or

more times without fungicide pressure to obtain sufficient material to carry out biological tests. Sporangia are collected and suspended in sterile water at 4 °C. Inoculation is performed by depositing fifteen 10–20 µl droplets onto the abaxial face of each leaf. After inoculation, leaves in Petri dishes are kept in the dark overnight at 22 °C with a 16 h day^{-1} photoperiod; the next day droplets are sucked up. After 6 or 7 days, freshly produced sporangia are harvested to inoculate the test units (Chen et al. 2007; Andrieu et al. 2001; Sirven and Beffa 2003; Genet and Vincent 1999).

To create a collection, field isolates of *P. viticola* may be collected from grape leaves from different sites in various countries, and single sporangiophore isolates may be produced from this mass by picking sporangia from sporangiophores with a fine needle (Blum et al. 2010a) or by release of sporangia on water agar and picking them and transposed them onto leaf disks (Corio-Costet et al. 2011).

16.2.3 Which Biological Test Can Be Used?

While there are many testing procedures, none seems to affect the conclusions concerning resistance. However, it is better to have standardized methods in order to compare results from different monitoring sessions. Before monitoring, it is generally best to use a range of fungicide concentrations to determine a discriminant dose.

16.2.3.1 Powdery Mildew: *E. necator*

According to the fungicide under study, biologic tests may vary. For example, since DMIs have no effect on spore germination, it is pointless to perform a germination bioassay. On the other hand, a test based on mycelium length or growth bioassay (including sporulation) is better suited for leaves.

16.2.3.1.1 Test of Mycelium Extension

A rapid test of mycelial extension can be performed with conidia being deposited on fungicide-treated or untreated leaf disks and then measuring the length of mycelium 72 h after inoculation. This test is efficient for comparing populations. Leaf disks are taken from each treatment and the germinating conidia removed with cellotape, stained with lactophenol cotton blue, and fixed on glass slides for microscopic observations of conidial germ tube length and morphology. Generally, a minimum of 100 germinating conidia showing a germ tube length of more or less 250 µm are counted (Cartolaro and Steva 1990; Thind et al. 1998). Untreated inoculated leaf disks served as controls.

16.2.3.1.2 Fungicide Sensitivity Assays (Growth and Sporulation)

Many authors have reported assay methods (Cartolaro and Steva 1990; Debieu et al. 1995; Erickson and Wilcox 1997; Délye et al. 1997b). Briefly, leaf disks of a susceptible cultivar are prepared as described in paragraph 2.2.1. Eight disks of eight different leaves are placed in Petri dishes (adaxial surface facing upward). A range of fungicide doses can be applied by spraying disks and incubation for 1 or 24 h before inoculation (Délye and Corio-Costet 1998) or by imbibition on a filter paper impregnated with 3 ml of water (untreated control) or 3 ml of fungicide solution (abaxial surface). After 30 min or 24 h, the disks are turned over and placed in Petri dishes and dried before inoculation (Steva and Clerjeau 1990; Debieu et al. 1995). In another technique, leaf disks are submerged for 1 h with regular agitation in a glass beaker containing fungicide solution and then dried between paper towels (Wong and Wilcox 2002).

After treatment with the various fungicide concentrations, leaves or disks are inoculated homogeneously with conidia at the rate of 600–800 spores by cm^2 with a single isolate or a population using a settling tower, and incubated in a growth chamber at 22 °C (Cartolaro and Steva 1990; Debieu et al. 1995), or are inoculated at a single point with two or three conidial chains (15–30 conidia) from an isolate (Wong and Wilcox 2002). After 12 or 14 days, the percentage of leaf surface infected is determined by scoring between 0 and 10 to indicate the surface area under attack and the degree of sporulation. Alternatively, observation is performed after 7 days of mycelial extension from the point of inoculation using an ocular micrometer and a stereomicroscope. The data are presented graphically by fitting a negative logistic regression curve, and the EC_{50} or EC_{90} and MIC are determined for each isolate or population (Fig. 16.2).

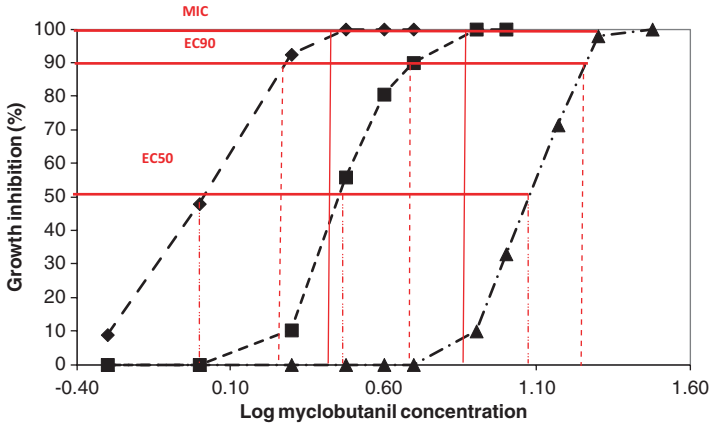

Fig. 16.2 Example of dose response curves to myclobutanil, a DMI fungicide, of three isolates of powdery mildew collected in France in 2007

16.2.3.2 Downy Mildew: *P. viticola*

Often the biological assays are not performed directly on the sample. After subculturing on new leaves in Petri dishes and incubation for 7 days, the new inoculum is used to test the sensitivity of isolates. With fungicide acting directly on the germination or motility of zoospores, it is possible to test a range of concentrations to assess the distribution of sensitivity. Such is the case with fungicide resistance that is recessive (e.g., CAA) or for detecting new forms of resistance and identifying whether they are disruptive or progressive (e.g., QiI, benzamide).

Various methods for testing fungicides can be used:

- One method is based on floating disks (Clerjeau and Simone 1982) in which 8 disks are cut and fungicide solutions dispensed into Petri dishes (20 ml/dish). Leaf disks are placed abaxially on water or fungicide solution. Each disk is inoculated with one droplet (25 µl) of sporangial suspension (20,000–200,000 spores ml^{-1}). Then, the inoculated disks are incubated at 22 °C with 14 h light $day^{-1}$and the droplets dried.
- A second method involves application of fungicides by droplets of 15 µl containing 1 volume of fungicide and 1 volume of sporangia at 200,000 spores ml^{-1} on the abaxial face of the leaf disks. Each Petri dish contains 10 disks from different leaves with 3 droplets (15 µl at 100,000 spores ml^{-1} final concentration). After incubation in the dark at 22 °C for 24 h to promote stomata opening and zoospore encystment, the droplets are sucked up, and scoring is performed 7 days after inoculation with a visual scale. The average of the 30 observations (10 disks × 3 droplets) is used to calculate the relative inhibition and to determine EC_{50} and MIC concentrations (Magnien et al. 2012).
- In a third method, disks are sprayed with fungicide solution (2 ml/Petri dish) (Herzog and Schuepp 1985).
- A fourth method involves inoculated disks by placing one drop (5 µl) of inoculum (40,000 sporangia ml^{-1}) mixed with various concentrations of fungicides on each disk (Reuveni 2003).

Inhibition of sporangia production can be assessed by counting with a hemocytometer or with a Coulter counter.

16.2.3.2.1 Sensitivity Test on Sporangia

To perform monitoring, discriminant doses of a fungicide (e.g., 10 mg to 1,500 mg l^{-1} of famoxadone) may be applied. For sensitive populations, or isolates that do not grow at fungicide concentrations higher than 10 mg l^{-1}, other concentrations are applied (from 0.1 mg to 10 mg l^{-1}). Fungicides are sprayed using a handheld sprayer onto the lower side of 10 grapevine leaf disks (Wong and Wilcox 2000; Chen et al. 2007; Herzog and Schuepp 1985), 1, 12, or 24 h before inoculation depending on the test. The disks are inoculated with 3 droplets of 10 µl with an inoculum of 20,000 or 10,000 sporangia per ml, and 12 h later, droplets are sucked up. It is

possible to inoculate with 50,000 sporangia per ml, leaf disks beforehand, placed on water agar in 24-well plates and treated 12 h before inoculation with different concentrations of a QoI (Sierotzki et al. 2005). After 7 days of incubation, the development of mildew on each disk is visually estimated as the proportion of leaf area with sporulation (Genet et al. 1997). For each concentration, the average score is converted to a percentage of inhibition by comparison with untreated disks and the EC_{50} and MIC determined. Different classes can be identified according to their sensitivity to a fungicide (e.g., famoxadone: sensitive isolate MIC < 10 mg l^{-1}) and highly resistant (MIC > 100 mg l^{-1}) (Chen et al. 2007). Depending on the authors, the fungicides used are either the commercial products, and the concentrations are calculated in terms of a.i. (Chen et al. 2007) or technical compounds dissolved in, e.g., 0.1 % of DMSO or acetone (0.2 %) with 0.005 % Tween 20 (Wong and Wilcox 2000; Sierotzki et al. 2005).

It is also possible to examine the effect of fungicide on zoospore release, motility, and integrity and on direct germination of sporangia by incubation of sporangial suspensions at 2.5×10^4 to 5×10^4 sporangia per ml with fungicides at various concentrations in 96-well microtiter plates at 20 °C for 24 h. Sporangia germination, zoospore motility, and zoospore integrity are calculated as a percentage compared to untreated sporangia (Andrieu et al. 2001; Blum et al. 2010a). To examine the development of haustoria, infected leaf pieces are cleared with boiling alcoholic lactophenol for 15 min and are transferred to alcoholic lactophenol containing 0.7 % of aniline blue and boiling again (10 min). Pieces are incubated in the staining solution until cold. Finally, the stained samples are removed and placed in saturated chloral hydrate solution for at least 1 day. Mycelial development is related to the number of haustoria formed per infection site after observations under a microscope (Herzog and Schuepp 1985).

16.2.3.2.2 Test on Oospores

Resistance to QoIs provides a good example. As the genetic basis of the resistant allele G143A is mitochondrial, it is worthwhile observing resistance either on oospores (year N) (Toffolatti et al. 2007) or on the first lesion arising from macroconidia germination (year N + 1) (Chen et al. 2007). Leaves showing mosaic symptoms at the end of the growing season (August–October) are randomly collected from vineyards. Three nylon bags per plot (pore size 100 μm), each containing 50 leaf fragments with oospores, are overwintered in the vineyards (Toffolatti et al. 2007). Germination test of oospores is carried out from January, and they are separated from the leaves by filtration through two nylon filters (100–45 μm) and resuspended in water. Oospore germinability is then assessed on 1 % water agar at 20 °C. Oospores are inoculated onto water agar containing fungicide (e.g., 1 mg l^{-1} azoxystrobin) in the dark for 14 days and observed under a microscope in order to assess the number of germinated oospores in comparison with oospores on untreated water agar. The oospores able to germinate on medium containing fungicide are considered as resistant. The percentage of resistant oospores is calculated as the

formula $G_{treated} \times 100/G_{untreated}$, and there is a good correlation between percentages of resistant oospores and the presence of the resistant allele G143A.

16.2.4 Baseline and Discriminant Dose

Before identifying the emergence and the presence of resistance (Fig. 16.1), it is imperative to know the sensitivity levels of pathogen populations. Usually, classes of sensitivity frequencies of natural populations with various doses of fungicides are assessed according to the geographical origin of populations or isolates. The results obtained may differ depending on whether one is dealing with mono-allelic disruptive resistance (e.g., QoI resistance) or pluri-allelic progressive resistance, be it mono or polygenic (e.g., DMI resistance). Commonly, fungicide sensitivity follows a Gaussian curve, but sometimes, there are outlier individuals (case of QoI fungicide) (Fig. 16.3). It is also possible to use isolates of laboratory collections to obtain baseline sensitivity. However, the best baseline is obtained from recently collected field isolates.

To determine the baseline, EC_{50} and MIC for pathogen isolates are estimated, by sensitivity bioassays with a range of fungicide doses, and then a discriminant dose (DD) is established for monitoring (Russell 2007). The DD commonly begins with a dose five- to tenfold higher than the MIC average of the pathogen population to detect the stage of emergence (Fig. 16.2) and even more (e.g., 100-fold for QoI resistance) or at least tenfold higher than EC_{50}. After detection and obtaining EC_{50} and MIC on resistant isolates, it is possible to develop a new DD to monitor the evolution of resistance and its maintenance if the fungicide resistance is progressive. EC_{50} is a good value to estimate resistance and is often better than the MIC or the EC_{90}. However, it should be determined with a number of isolates and/or

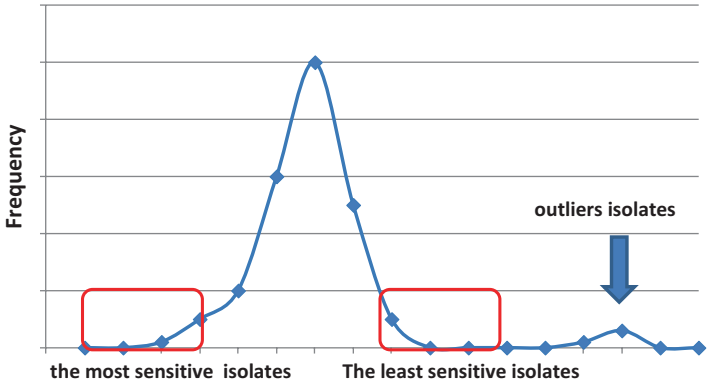

Fig. 16.3 Distribution of sensitivity of pathogen to fungicides (e.g., QoI) with a Gaussian distribution and some isolates that are outliers. This is typical of resistant isolates already naturally present in the field before the use of a fungicide

populations sampled from various vineyards to provide enough variability. During these efficacy tests, it is always important to observe differences in growth rate, sporulation, the aspect of the mycelium, and the pathogenicity of the isolates. These data provide information on the fitness of the resistant isolate collected in the vineyard.

16.2.4.1 EC_{50} and MIC

A range of fungicide concentrations (6–10) is sprayed and allowed to dry before inoculation as described in 2.3. The percentage of leaf tissue infected with powdery mildew is evaluated, and the data converted to EC_{50} values or MIC. Measurements are made for each fungicide concentration, and the mean values for eight replicates of leaf disks per treatment are used to calculate the relative growth and then the efficacy, according to the formula:

Efficacy = 100 × [Cont-Fung/Cont], with **Cont,** which is the average of attack on untreated disks, and **Fung,** that on treated disks. Dose response curves for isolates or populations are generated by plotting the relative growth values against the $\log_{10}$ of the fungicide concentration used, and the $\log_{10}$ effective dose to reduce growth by 50 % (EC_{50}) is calculated from the regression equation generated through the linear portion of the sigmoid curve or graphically (Fig. 16.2). The minimal inhibitory concentration (MIC) and EC_{90} can also be calculated (Wong and Wilcox 2000, 2002; Délye and Corio-Costet 1998; Sombardier et al. 2010).

16.2.4.2 Resistance Factor

A comparison of isolate (or population) sensitivity to fungicide based on the EC_{50} and the MIC of various sensitivities makes it possible to obtain a resistance factor (RF). The resistance factor is calculated for isolates displaying decreased sensitivity to fungicide as follows: $RF = EC_{50R}/EC_{50}s$, where EC_{50R} is the EC_{50} value for isolates or populations studied and the EC_{50S} is the mean of EC_{50} for all reference sensitive isolates or populations. According to the size of this RF and the diversity of the pathogen population, it is possible to estimate the risk of loss of efficacy in the field (Fig. 16.4).

16.3 Monitoring in the Vineyard

16.3.1 Monitoring for Progressive Resistance (DMI Resistance) in E. necator

DMI fungicides inhibit sterol biosynthesis and represent an important fungicide family and include up to five different chemical families (Worthington 1988) and more than 18 molecules. These fungicides have been very successful in view of the low

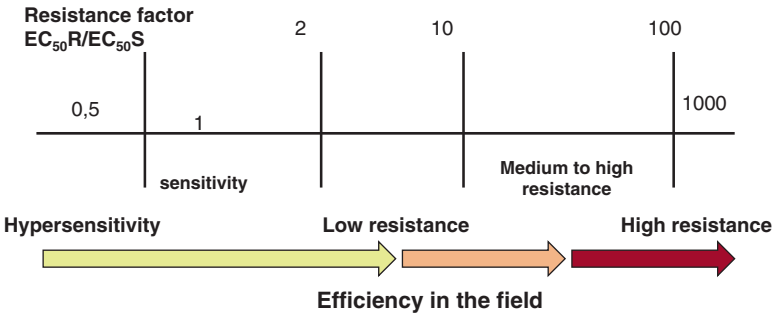

Fig. 16.4 Evolution of resistance factor linked to efficacy of fungicides in the vineyard. *Yellow arrow* = effective treatments, *beige arrow* = effective treatments with sometimes loss of efficiency, *brown* = loss of efficiency in the field

doses used (from 17 to 100 g/ha) and their penetrating and systemic properties. They all inhibit eburicol-C14-demethylase, an enzyme involved in methylene-24-cholesterol biosynthesis, the major sterol of *E. necator* (Délye and Corio-Costet 1994).

Monitoring DMI sensitivity identified the first case of resistance in grape powdery mildew in Portugal in 1984 (Steva et al. 1989), then in France in 1988 (RF varying from 2–50 to triadimenol) (Steva and Clerjeau 1990), and in Italy (RF = 6 for fenarimol) (Aloi et al. 1991) (Table 16.2).

Introduced in 1982 in California, triadimefon was effective until 1986, and resistance was officially described in 1988 (Ogawa et al. 1988). From the 1990s, resistance toward 5 molecules was identified in Austria (Redl and Steinkellner 1996). Myclobutanil resistance was observed in the New York state 6 years after its introduction, and in Australia, the RF varied from 0.6 to 50. From 2000, all vineyards seem to be affected by DMI resistance. Isolates and populations have been identified that exhibited variable RF from 2 to 180 (Table 16.2).

From the sensitivity baselines (EC_{50}, MIC), it is possible to carry out monitoring with discriminant doses of the order of 1–10 mg l^{-1} (a.i.) for each fungicide, showing that all the isolates exhibiting an RF (>5) have lost some of their sensitivity (Fig. 16.4). The gene (*cyp 51*) coding for the target enzyme was sequenced and the presence of one allele involved in the resistance identified (Délye et al. 1997c, d, 1999). A single modification at codon 136 of the *cyp 51* gene replaced a phenylalanine residue with a tyrosine (Y136F, Délye et al. 1997c, d) and which correlated perfectly with RF to triadimenol greater than or equal to 8. Identification of this allele, added to biological assays, helps assess the evolution of resistance in the field and give quantitative results (Dufour et al. 2011) to edit national mapping of resistance status of vineyards or the global evolution of resistance each year (Fig. 16.5). This helps making recommendations to vine-growers so that they can avoid the loss of efficacy of fungicides (Corio-Costet et al. 2014a, b; French national memorandum on mildews 2014). Monitoring of this allele marker by q-PCR (Fig. 16.5) showed an increase of the presence of the resistance marker in *E. necator* populations from 2008 to 2012, with a stability between 2010 and 2012, and even a decrease in

Table 16.2 Detection of DMI resistance in vineyards worldwide

Year[a]	Country	RF	Fungicide	References
1984	Portugal	2–140	Triadimenol	Steva et al. (1989)
1986	USA (California)	0.3–180	Triadimenol, Myclobutanil, Fenarimol, Tebuconazole, Triflumizole	Boubals (1987), Ogawa et al. (1988), Gubler et al. (1996), Erickson and Wilcox (1997), Ypema et al. (1997), Northover and Homeyer (2001), Miller and Gubler (2004), Wong and Wilcox (2002), and Colcol et al. (2012)
1997	USA (New York)			
1987	France	2–140	Triadimenol, Myclobutanil, Fenarimol	Steva and Clerjeau (1990), Debieu et al. (1995), Délye et al. (1997b), and Dufour et al. (2011)
1988	Turkey			Ari and Delen (1988)
1988	Italy		Fenarimol, Triadimefon	Aloi et al. (1991)
1991	Switzerland			Pezet and Bolay (1992)
1995	Austria	1–6	Penconazole, Pyrifenox, Myclobutanil, Triadimenol	Redl and Steinkellner (1996) and Steinkellner and Redl (2001)
1995	Australia	0.6–50	Triadimenol	Savocchia et al. (2004)
1997	India			Thind et al. (1998)
2000	South Africa	1–48	Penconazole, Flusilazole, Triadimenol	Halleen et al. (2000)

[a]Date of first observation of loss of efficacy in the vineyard

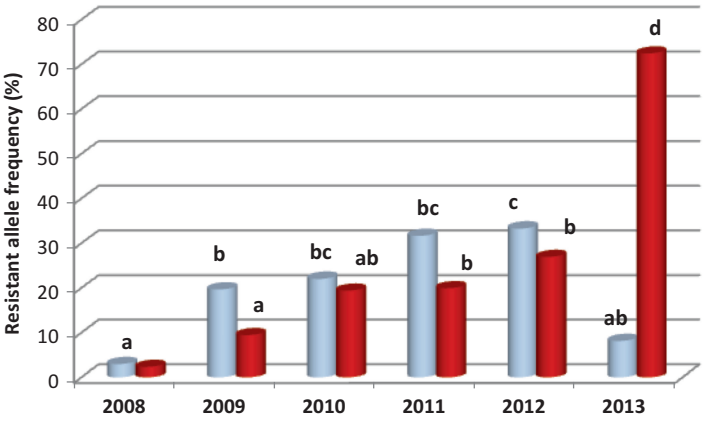

Fig. 16.5 Average regional frequencies of alleles conferring resistance to DMI fungicide (Y136F, in blue or gray) and to QoI fungicide (G143A, in *red* or *black*) in France, in *E. necator* populations between 2008 and 2013. The number of populations analyzed varied from 33 to 78 per year (From Corio-Costet et al. 2014a)

2013, which was partially due to the campaign to limit DMI use in vineyards since 2009, by never using them twice consecutively, and to be careful when treating. However, DMI resistance is polygenic, and besides modifications of the gene (*cyp 51*) coding for the target enzyme (C14-demethylase), there are other mechanisms of resistance (Stergiopoulos et al. 2003; Buchenauer 1995). Monitoring of the Y136F allele makes it possible to anticipate the evolution of the population and, when complemented with a discriminant dose of 5–10 mg L^{-1} of tebuconazole, confirms resistance, and treatment protocols can then be modified.

One problem with progressive polygenic resistance is the discrete onset of the phenomenon, which is closely linked to the mode of reproduction of the pathogen and the selection pressure exerted by the fungicide. This is particularly the case for the genetic group B that survives during the winter either in the dormant buds or by sexual reproduction. Group B contains the majority of isolates on which fungicide selection is applied. On the contrary, genetic group A in France survives essentially as dormant mycelium and undergoes little fungicide pressure (Corio-Costet 2007). Estimating population diversity before and during monitoring can also be as important as having good molecular tools for detection or a good test of discrimination.

16.3.1.1 False Positive: Case of Sympatric Populations of *E. necator* in the Vineyard

It is possible to detect changes in sensitivity and the beginning of resistance (Table 16.3), but interpretation errors due to pathogenic diversity can occur. For example, mildew samples collected in vineyards in the South of France, before flowering, and with young leaves totally mildewed from budbreak (Corio-Costet 2007; Amrani and Corio-Costet 2006; Steva 1991) were sensitive to tebuconazole (mean EC_{50} of 0.12 mg $l^{-1)}$. A second sampling from the same area 6–8 weeks later (after flowering) considered mildew as resistant (mean EC_{50} of 0.73 mg l^{-1}, Table 16.3), and an RF of 6 between the two samplings suggested disruptive resistance. In fact, monitoring was applied to two sympatric populations belonging to two different genetic groups, one more sensitive than the other one (Délye et al. 1997a, b; Corio-Costet et al. 2003). None could really be considered as resistant when compared to the baseline sensitivity of *E. necator* isolates. Resistant isolates collected in the same area had a mean EC_{50} of 5.84 mg l^{-1}. Simply, group A isolates are more sensitive than group B isolates (Corio-Costet et al. 2003; Miazzi and Hajjeh 2011).

16.3.1.2 Cross-Resistance

Resistance to one DMI fungicide can confer positive cross-resistance, i.e., resistance to triadimenol also leads to resistance to other molecules such as myclobutanil, fenarimol, penconazole, or cyproconazole (Erickson and Wilcox 1997; Délye et al. 1997b). In South Africa in 1989, *E. necator* isolates showed reduced sensitivity

Table 16.3 Example of variation in sensitivity of *E. necator* isolates to triadimenol or tebuconazole

	Isolates	Year of sampling	$EC_{50} \pm SE$ (mg l^{-1})	$EC_{50} \pm SE$ average (mg l^{-1})	RF
Triadimenol					
Sensitive isolates	Coc2	1988	0.11 ± 0.02	0.06 ± 0.045	
	Moa2	1989	0.02 ± 0.00		
	Aor3	1990	0.05 ± 0.01		
Resistant isolates	Moa5	1989	3.58 ± 0.45	5.06 ± 1.75	84 (R/S)
	Aor2	1990	4.57 ± 0.22		
	Coc5	1990	7.13 ± 0.22		
	Mr6	1993	3.28 ± 0.37		
	Mr3	1994	6.47 ± 0.19		
Tebuconazole					
Genetic group A*	10 sensitive isolates (SA)	1998		0.12 ± 0.02	1
Genetic group B*	10 sensitive isolates (SB)	1998		0.73 ± 0.33	6 (SB/SA)
Genetic group B	10 resistant isolates (RB)	1998		5.84 ± 0.73	8 (RB/SB) or 48 (RB/SA)

From Steva (1991), Délye (1997), and Corio-Costet et al. (2003)
* = sensitive isolates without resistant allele in position 136 (Y136T), considered as sensitive
RF resistant factor, *SB* sensitive (SB) or resistant (RB) isolates belonging to genetic group B. *SA* sensitive isolates belonging to genetic group A

to triadimenol, flusilazole, and penconazole (Halleen et al. 2000), but the different combinations found between the three fungicides corroborated the polygenic nature of DMI resistance. However, in France, among more than 175 isolates tested with seven DMIs, none was resistant to more than 5 of these fungicides. Recently in Virginia, the comparison between sensitive isolates and isolates from vineyards treated with DMIs showed RF varying from 31 (triflumizole) to 144 (myclobutanil), with RF of 53, 75, and 45, for fenarimol, tebuconazole, and triadimefon, respectively (Colcol et al. 2012).

16.3.2 Monitoring in the Vineyard for Disruptive Resistance: Case of QoI Fungicides

The market launch of successful molecules inhibiting mitochondrial respiration at the end of the 1990s, such as the QoIs (quinone outside inhibitors), highlighted a particular case of resistance, although the genetic support (cytochrome b gene) is mitochondrial. Commonly from 1997, the existence of *P. viticola* populations showing a positive cross-resistance to QoIs (azoxystrobin, pyraclostrobin, kresoxim-methyl, trifloxystrobin, famoxadone, and fenamidone) has been described (Heaney

et al. 2000; Jordan et al. 1999; Hollomon et al. 2005; Gisi et al. 2002). Baselines quickly revealed the existence of isolates or populations 100–1000 times less sensitive than sensitive populations of both *E. necator* and *P. viticola* (Heaney et al. 2000; Sierotzki et al. 2005, 2008; Baudoin et al. 2008; Chen et al. 2007; Dufour et al. 2011; Corio-Costet et al. 2008; Corio-Costet 2012; Toffolatti and Vercesi 2012; Sirven et al. 2002). These compounds are recommended for preventive applications. Some are systemic and are capable of circulating via the translaminary route and via the vascularization, which partially explains their strong action on sporulation (Gisi et al. 2002).

16.3.2.1 QoI Fungicide Resistance in *P. viticola*

The QoI fungicides possess strong anti-oomycete activity at low doses (from 50 to 400 g ha^{-1}). They have good persistence and strongly limit differentiation of spores and the release of zoospores (Reuveni 2001; Andrieu et al. 2001). Complete inhibition of the sporulation is obtained with trifloxystrobin at 5 mg l^{-1} applied preventively on young inoculated plants (Reuveni 2001). A dose of famoxadone between 0.01 and 0.1 mg l^{-1} is sufficient to lyse the zoospores of *P. viticola* (Andrieu et al. 2001).

Similar results have also been obtained with azoxystrobin, showing an average EC_{50} of 0.04 mg l^{-1} for 81 isolates, with values ranging from 0.05 to 0.94 mg l^{-1} (Wong and Wilcox 2000). The distribution of sensitivity to famoxadone in 103 European populations of *P. viticola* was described as unimodal with a mean EC_{50} of 0.029 mg l^{-1} and variations ranging from 0.001 to 0.15 mg l^{-1} (Genet and Vincent 1999).

Hence, like other single-site fungicides, the interesting properties of these molecules (wide spectra of efficacy, curative effect) have quickly led to a strong selection process. This in turn has led to the appearance of resistance not only in French vineyards (Magnien et al. 2003, 2012) but also in other vineyards worldwide (Brunelli et al. 2003; Baudoin et al. 2008; Corio-Costet et al. 2008, 2011; Furuya et al. 2010; Heaney et al. 2000; Gullino et al. 2004; Gisi et al. 2000; Sierotzki et al. 2008).

In most cases, resistance is attributed to the presence of a major mutation in the mitochondrial cytochrome b gene, leading to the substitution of a glycine by an alanine at position 143 (G143A), including grapevine downy and powdery mildew (Gisi et al. 2002; Grasso et al. 2006; Chen et al. 2007). Another mutation can add to G143A and involves an alteration of codon 129, where a phenylalanine residue is replaced by a leucine (F129L), even if this modification confers only a low level of resistance (Gisi et al. 2002). As QoI resistance is correlated with the presence of allele G143A in the vineyard, various molecular tests have been developed after identification of discriminant doses in order to identify the resistant isolates or populations. These tests are based on PCR-RFLP (Corio-Costet et al. 2011; Chen et al. 2007; Baudoin et al. 2008; Furuya et al. 2010) or on quantitative PCR and quantification (Sirven et al. 2002; Sirven and Beffa 2003; Collina et al. 2005; Sierotzki et al. 2005, 2008; Toffolatti et al. 2007).

Studies of the gene coding for the target enzyme (*CYT bc1*) have highlighted the existence of two different genetic mitochondrial haplotypes (I and II), demonstrating that at least two independent events led to QoI resistance in European populations (Chen et al. 2007). The same study also showed the existence of several possible subspecies of *P. viticola* in American populations.

Biological assays carried out on bulk samples provide qualitative information if a discriminant dose is used. Another test developed for assessing QoI resistance is based on the germinability of oospores at discriminant doses. A reference dose for resistant field isolates is 10 mg l^{-1} (Heaney et al. 2000; Chen et al. 2007; Toffolatti et al. 2007).

In France, samples are dispatched by a national surveillance network and are accompanied by information index cards clarifying the treatment programs, locality, cultivar, and if losses in efficacy have been observed. Data for the previous years in the vineyards concerned as well as the rate of damage are also noted. Tests are performed either from lesions of *P. viticola* on leaves or grapes or from new sporulations, which are obtained in wet chambers after 24 h of incubation.

In 2005, QoI resistance quickly reached a mean of 29 %, but it varied significantly from one vineyard to another (from 0 to 70 %) (Corio-Costet et al. 2008; Fig. 16.6). In Champagne vineyards, similar results have been obtained with an average of 33 % of resistant lesions and a range from 0.01 to 77 %. A study on diversity performed with microsatellite markers (Delmotte et al. 2006) showed that the genotypic diversity is smaller when the frequency of resistance exceeds 30 % and that this diversity tends to decrease during the season in the resistant plots (Corio-Costet et al. 2008). This decreasing diversity of resistant populations was also demonstrated on plots treated with azoxystrobin (Matasci et al. 2008). In plots not having received any treatment with QoIs, the frequency of the resistance allele G143A in oospores populations was 10 %. Conversely, prevalence reached 90 % in plots treated 5–6 times (Toffolatti et al. 2011).

The persistence of resistance should also be monitored (Fig. 16.1), and various studies show that QoI resistance is rather persistent. All isolates are collected between 2002 and 2007 under fungicide selection pressure or not remained resistant. Furthermore, a study on the fitness of resistant isolates of *P. viticola* during the asexual phase demonstrated that QoI-resistant isolates exhibit good fitness (germination, sporulation, and infection frequency) and that they are competitive (Corio-Costet et al. 2011). Nonetheless, some studies show that after sexual reproduction, a decrease in QoI resistance occurs at the beginning of the season when there is no selection pressure (Toffolatti et al. 2007). In Brazil in 2002 after several years of use of QoI fungicides, the allele (G143A) frequency reached 100 %. By interrupting the application of QoI fungicides for 2 years, the resistance frequency declined to 2 %. On the other hand, 5 consecutive applications led to resurgence of resistance frequency of 100 % during the season, and it decreased again in 2005 (Sierotzki et al. 2008). Indeed, since the mutation is in a mitochondrial gene, this suggests that there is a maternal mitochondrial heritability. According to whether mitochondria “females” carry the resistant allele or not,

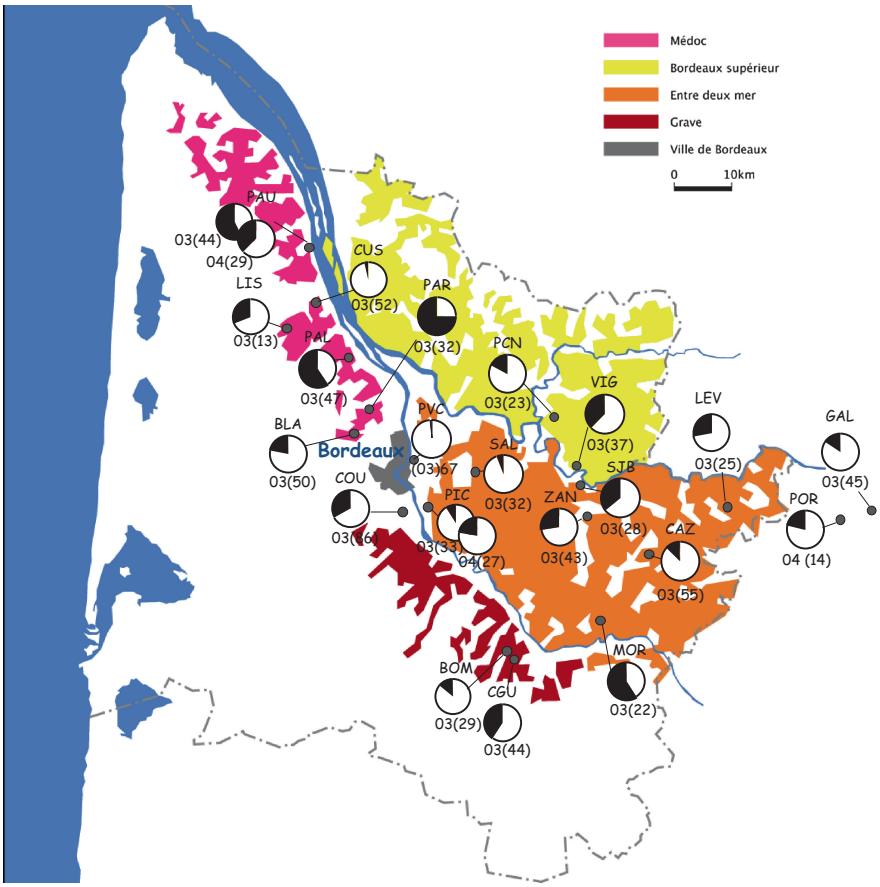

Fig. 16.6 Frequency of QoI fungicide resistance among 22 populations of *P. viticola* in Bordeaux vineyards collected in 2003 and 2004. Within the circle, QoI fungicide-resistant isolates are presented in black and sensitive isolates in white. The number of isolates analyzed is in brackets. Bioassays were performed with a discriminant dose of 50 mg l^{-1} of famoxadone and detection of allele G143A by PCR-RFLP (Chen et al. 2007)

one may expect a "dilution" of resistance phenomena, even its regression after every sexual cycle (Corio-Costet et al. 2011). This also suggests that resistant isolates might be less fertile or that resistant ones (Sierotzki et al. 2008).

16.3.2.2 QoI Fungicide Resistance and *E. necator*

The first QoIs used in the vineyard against *P. viticola*, such as famoxadone or trifloxystrobin, were also partially effective against *E. necator* and favored the selection of QoI resistance in *E. necator.* Methods used are similar to those described in

paragraph 2.3, but as QoI fungicides act on respiration and can inhibit germination, a germination bioassay can be performed (Reuveni 2001; Miles et al. 2002).

16.3.2.2.1 Germination Bioassay

The test is performed on microscope slides covered with 3 ml of water agar amended with the commercial formulation of the fungicide at different concentrations (e.g., from 0.0001 to 100 mg l^{-1}). The medium can be also supplemented with 100 mg l^{-1} of salicylhydroxamic acid (SHAM) to block the alternative oxidase (AOX). After 24 h inoculation (conidia transferred with sterilized paintbrush), slides were observed under fluorescent light and 50 conidia observed for germination (Miles et al. 2002). All isolates with germination inhibited by trifloxystrobin at 1.1–100 mg l^{-1} exhibited the resistant allele G143A. A concentration of 0.1 mg l^{-1} inhibited germination of *E. necator* conidia of sensitive isolates completely (Reuveni 2001).

16.3.2.2.2 Discriminant Doses and Monitoring

Wong and Wilcox (2002) described the baseline of *E. necator* sensitivity to azoxystrobin from samples collected in 1999 from untreated vineyards. EC_{50} values ranged from 0.0037 to 0.028 mg l^{-1} with a mean of 0.097 mg l^{-1}. In California, EC_{50} obtained in 2002 varied from 0.00003 to 0.343 mg l^{-1} of trifloxystrobin (Miller and Gubler 2004). A concentration of 0.1 mg l^{-1} inhibited germination of *E. necator* conidia completely (Reuveni 2001). In European vineyards, the first case of resistance was identified in 2007 in Hungary (Bencené pers. comm), and resistant isolates were first detected in French vineyards in 2008 (Dufour et al. 2011).

However, it was not difficult to design allele-specific molecular tools based on the presence or not of the mutation at codon G143A. Therefore, q-PCR tools were soon used to detect and monitor the evolution of resistance in *E. necator* populations in the United States (Baudoin et al. 2008) and in France from 2008 (Dufour et al. 2011). It was noticed that samples exhibiting the QoI-resistant allele frequently also had the DMI-resistant allele. Overall, the mean percentage of QoI-resistant alleles nationwide for 2008 and 2009 reached 5.29 % (Fig. 16.5). However, from 2010, the mean percentage increased to 18 %, then 37 % in 2012, and 72.4 % in 2013. This shows that in spite of recommendations to limit the use of QoI fungicides, both in solo applications and in mixtures, in the French vineyards, they are still used, and the selection continues to be applied on powdery mildew populations. Even moderate, the use of QoI fungicide has rapidly led to resistance selection in *E. necator* in the field in Hungary (Taksonyi et al. 2013).

16.3.3 *Monitoring for Recessive Resistance: Case of CAA Resistance in* P. viticola

The carboxylic acid amide (CAA) fungicides, including dimethomorph, iprovalicarb, benthiavalicarb, mandipropamid, and valifenalate, are single-site fungicides inhibiting cell wall biosynthesis of oomycetes by inhibition of 1,3-β-glucanase exerting a glucanosyltransferase activity (*PiCesA3* and *PvCesA3* genes) in cellulose biosynthesis in *Phytophthora infestans* and in *P. viticola* (Blum et al. 2010a, b). The mechanism of resistance involving a recessive mutation in the *PvCesA3* is discussed in Chap. 20, but other recessive genes may also be involved. Consequently, resistance of *P. viticola* to CAAs might be reduced if appropriate strategies for commercial fungicides use were implemented in the field (Gisi 2012). Indeed, FRAC and the French national memorandum (2014) recommend the use of CAAs in mixtures with multisite fungicides or other non-cross-resistance groups of fungicides.

At the beginning of CAA use (e.g., dimethomorph), EC_{95} values ranged from 0.25 to 1.15 mg l^{-1} (Bissbort and Schlosser 1991), but this fungicide was used on average only twice per year. When new CAA fungicides entered the market 6–8 years later, EC_{50} values of iprovalicarb showed a wide range of concentrations from <1–30 mg l^{-1}, with a mean of 2 mg l^{-1} (Suty and Stenzel 1999). In 2003, the mean EC_{50} of dimethomorph in the French vineyard was 5.92 mg l^{-1}, i.e., fivefold higher than the EC_{50} found in 1991. In the same isolate collection, iprovalicarb sensitivity showed a mean EC_{50} of 78.7 mg l^{-1} (Corio-Costet 2012), suggesting the appearance of isolates resistant to iprovalicarb and dimethomorph. The EC_{50} for isolates sensitive to CAA fungicides is 5–100 times lower than those of resistant isolates (Table 16.4). Monitoring carried out between 2005 and 2010 on more than 590 French downy mildew populations showed a decrease in the sensitivity of these populations from 74 to 11 % (Fig. 16.7), with a more or less stable intermediate group during the monitoring period and an increase in resistant populations, with a shift from 9 to 32 % (Magnien et al. 2012). However, these results hide a large disparity in resistance behavior according to region and disease pressure. As downy

Table 16.4 CAA sensitivity of *P. viticola* isolates

Fungicides	Mean of EC_{50}[a] in sensitive isolates (mg l^{-1})	Mean of EC_{50} in resistant isolates (mg l^{-1})
Dimethomorph	10.3[b]	>100
Iprovalicarb	6.2	>30
Mandipropamid	0.95	>300
Benthiavalicarb	1.10	>300

[a] Concentration of fungicide inhibiting 50 % *P. viticola* growth
[b]The mean EC_{50} was 0.21 mg l^{-1} in 1989

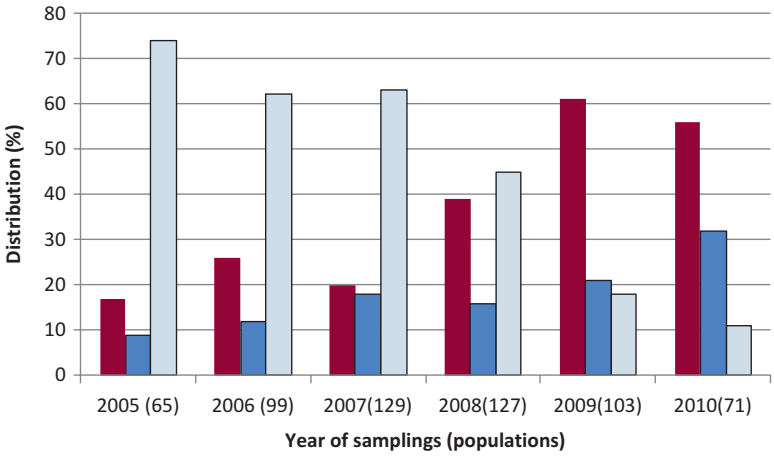

Fig. 16.7 Evolution of CAA resistance in *P. viticola* populations from 2005 to 2010 (From Magnien et al. 2012). *Light blue*, sensitive; *blue*, medium sensitive; *brown*, resistant isolates. Bioassays were performed with discriminant doses of 10 and 100 mg l^{-1} of dimethomorph

mildew populations in French vineyards are now resistant, the efficacy of treatments based on CAA fungicides is directly related to the type and dose of the associated product.

Monitoring can also be used to quantify the number of zoospores encysted in germination with a discriminant dose (e.g., 30 mg l^{-1} for iprovalicarb) and for establishing the resistance level according to the resistant spores (Latorse et al. 2006).

16.4 Conclusion

Data obtained by large-scale sampling on various plots of land in various regions, or in more restricted on trial plots where the application of a fungicide is repeated, provide information about the risk of the emergence of resistance and on the extent to which resistance to a given fungicide may become more widespread. All the data acquired at the regional and national level are used to make recommendations on fungicide use to limit resistance. A national memorandum gives advice on the use of fungicides according to the risk of resistance. Nonetheless, it should be remembered that in the fight against *E. necator* or *P. viticola*, the available formulated products are different and that they are often used solo against powdery mildew or as a mixture against downy mildew.

The diverse monitoring methods provide different data on the sensitivity of isolates or populations, so the most appropriate methods need to be optimized and evaluated for each pathogen-fungicide system (Brent and Hollomon 2007a, b).

Their impact depends on the applicability of the test and the proportion of resistant isolates or populations, together with the characteristics of the targeted genetics, fitness, and biology.

The risk assessment of fungicide resistance depends on the mode of action of fungicide (single site or multisite) and on the genetic support of the target (nuclear or mitochondrial). Generally, the mitochondria genome is more plastic than the nuclear genome, and so the risk of resistance is often greater. To the risk depending on the mode of action of the fungicide should be added the risk connected with the pathogen. Indeed, according to its capacity to reproduce (sexual and/or asexual), the appearance and the spread of resistance will depend on the fungicide selection pressure exerted on the pathogens. Hence, in grapevine powdery mildew which is generally treated with a single product, the evolution of the resistance seems less rapid than that of downy mildew, which is treated with a mixture of fungicide. The FRAC group gives recommendations about the risk of resistance of different pathogens with various fungicides (Brent and Hollomon 2007a).

The dissemination of monitoring results and the communication between the public sector and industrial operators in the field are required, so that the data obtained to date can be interpreted correctly and that some recommendations can be made for the sustainability of the use of fungicides.

These measures will help decrease fungicide selection pressure on obligate pathogens and contribute to limiting the risk of resistance. Spraying should be performed according to the best possible practice (avoiding sub-dosages or untreated areas), and preventive measures should be set up to limit the development of pathogens. The French National Memorandum (2014) recommends the following:

> alternating the use of fungicides having a single-site mode action, with others that have another mode of action. It is important not to alternate different products having the same mode of action. For fungicides for which resistance is widespread such as QoI and CAA, it is advisable to make only one treatment per season.

The 2014 French National Memorandum on Mildew states that the use of QoI fungicides is no longer recommended because of widespread and persistent resistance. Consequently, the efficacy of products containing QoIs is often exclusively related to the type and dose of the associated multisite products used (e.g., copper, mancozeb, cymoxanil, folpet).

Could a new bioassay-based monitoring strategy be of use? At the moment, the QiI (quinone inside inhibitor, cyazofamid) controls the majority of *P. viticola* isolates (94 %) at a dose close to 1 mg l^{-1}. On the other hand, 6 % of isolates from various regions have an MIC higher than 100 mg l^{-1} (Magnien et al. 2012). As for QoIs, there is a Gaussian distribution of sensitivities (Fig. 16.3) with outliers (RF of 100), suggesting that these isolates occurred prior to the use of QiI fungicides, and if used more than once or twice per season, QiI resistance will quickly emerge.

The degree of variation in baseline sensitivity is therefore a good marker of changes in resistance. A wide variation in baseline sensitivity (factor 100 and more) can be interpreted as real resistance provided that the baseline was obtained from field populations of different origin beforehand so as to avoid misinterpretations. A

lower evolution of this baseline (RF from 5 to 10) does not necessarily indicate that the resistant isolates are leading to losses of efficiency. Nonetheless, some populations (already tested) which would slide gradually toward higher RF (e.g., DMI resistance or CAA resistance) in time allow the presence of a real risk of progressive or recessive resistance to be detected. Monitoring is also undertaken to investigate complaints from growers of an apparent loss of performance of a fungicide and to guide the selection of future fungicide treatments.

Unfortunately, one of the problems associated with the limitation of fungicide use and improvement of treatment programs is that monitoring is often stopped for financial reasons. Continuing to monitor for several years, even after resistance has been demonstrated, can lead to better understanding of how pathogenic populations adapt to the various pressures of selection exerted in the vineyard. Combining monitoring with the practices of growers (sub-dosages, inadequate adjustment of spraying equipment) can also improve understanding of the onset of resistance and its management. For this reason, monitoring of *E. necator* and *P. viticola* in France is now associated with meta-analyses taking into account of spraying equipment and applied doses. During the last 7 years, monitoring of mildews has shown that it has become more and more frequent to encounter isolates that are resistant to one or two fungicides with different modes of action and even three (DMI, QoI, quinoxyfen) in powdery mildew. In downy mildew, isolates resistant to three and even four different modes of action (e.g., CAA, QoI, mefenoxam, cymoxanil) are now encountered. It should now be made law to monitor the evolution of pathogen populations if they are to be controlled during the next decades, as should the use of other methods of control in association with fungicides (biological, breeding, plant defense stimulation, etc.). Biological tests remain bulwark in monitoring before the development of molecular tools.

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