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FUNGICIDE RESISTANCE IN GRAPEVINE DOWNY MILDEW MANAGEMENT: PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *PLASMOPARA VITICOLA* POPULATIONS FOR RESISTANCE TO FUNGICIDES

AGR/12 Plant Pathology

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Abstract

Viticulture plays a role of prime importance within the world agricultural panorama. In 2020 the total world area planted with vineyards for all intended uses (wine and juices, table grapes and raisins), including young vines and not yet in production, was estimated at 7.3 Million ha by the International Organization of Vine and Wine in 2020. Within this vast production panorama, the Eurasian grapevine (*Vitis vinifera* L.) is the most cultivated species of grapevine due to the high quality of its grapes. One of the main problems affecting this species is that it is highly susceptible to infections of the phytopathogenic oomycete *Plasmopara viticola* (Berk. *et* Curt.) Berl. & De Toni. This pathogen is native to North America and with favorable climatic conditions can cause considerable damages to the grapevine production both from a qualitative and a quantitative point of view. *P. viticola* is a polycyclic pathogen, able to carry out numerous cycles of infection during a single vegetative grapevine season. Consequently, given the high susceptibility of *V. vinifera* cultivars to this pathogen in areas with frequent rainfall and moderate temperatures during the growing season, the cultivation of traditional varieties is not conceivable without frequent applications of fungicides.

Fungi and fungal-like organisms, such as the oomycetes, are highly adaptable to different environmental conditions, to host defense mechanisms and to fungicide selection. Repeated treatments with selectively active, site-specific fungicides, is frequently followed by the development of the phenomenon of fungicide resistance, which represents one of the major threats for downy mildew control and for modern agriculture in general, because it potentially leads to a reduction of disease control in the field (practical resistance). In order to preserve the effectiveness of such compounds, fungicide resistance must be carefully managed, and to this purpose proper disease control strategies have to be implemented by reducing the selection pressure associated to the fungicide use. The implementation of sound anti-resistance strategies is based on many factors: the risk of a particular fungicide class to evolve resistance, the risk related to the pathogen features, the agronomic risk associated to specific climatic conditions and the results obtained in sensitivity monitoring activities, that allow to characterize the fungicide sensitivity of pathogen strains or populations through bio tests and molecular diagnostic tools. In particular, monitoring of *P. viticola* populations for their sensitivities to the different active principles plays a key role in fungicide resistance management. However, for some fungicides these data are currently not yet present or incomplete, and actual testing methodologies to investigate some life stages of the pathogen are quite limited because they can provide only a qualitative description of resistance status.

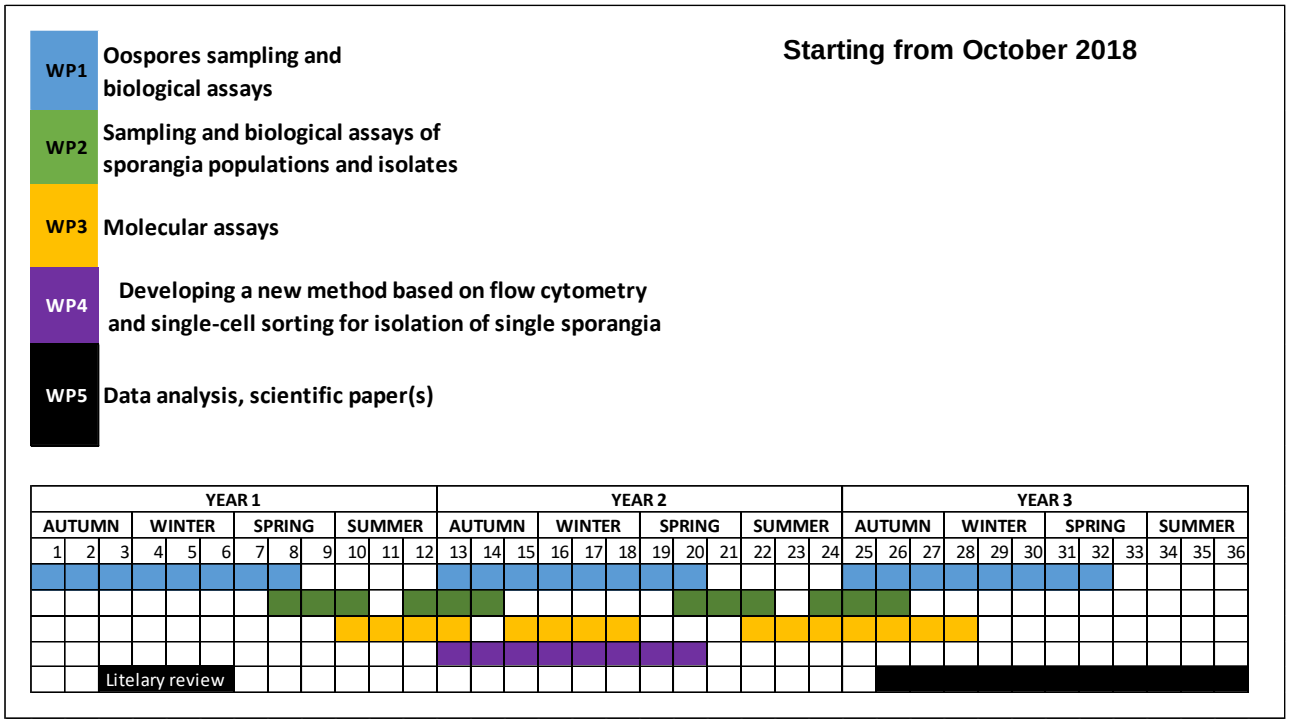
Considering these current challenges, the aims of my PhD project were: a) to evaluate the sensitivity profile of *P. viticola* populations to different fungicide classes subjected to different disease pressure levels and spray programs in open field; b) to develop a new method based on flow cytometry and single-cell sorting for isolation of single sporangia in order to obtain a precise estimation of the percentage of sporangia able to positively infect grapevine plants in the population; c) to characterize possible resistant strains for the mechanism of resistance and pathogenicity.

In general, among the populations tested we found a good sensitivity for the fungicides under investigation. Sporadic cases of resistance were detected, and for some of them it was possible to link the resistant phenotype to single point mutations in the gene encoding the target protein.

Depending on the life stages of the pathogen under investigation, more quantitative or qualitative data were obtained according to the testing methods available. This made it necessary to develop a further tool to make the investigations on the asexual reproduction stages of the pathogen more homogeneous to those of sexual reproduction. Flow cytometry and single-cell sorting have proven to be excellent technologies to bridge this gap, and the quantitative method here developed and proposed could be positively implemented in future for large-scale monitoring investigations of fungicide resistance.

The PhD project was set in 5 different work packages (WP), starting from October 2018 as reported in figure 1.

Figure 1: temporal declination of each work packages of the PhD project in weekly units



(WP1) Oospores sampling and biological assays. Sampling for *P. viticola* sexual spores (oospores) was carried out in Autumn of year 1 (months 1-3), 2 (months 13-15) and 3 (months 25-27). Sensitivity assays of oospores were carried out in spring of year 1 (months 4-8), 2 (months 16-20) and 3 (months 28-32).

(WP2) Sporangia population sampling and biological assays. Sampling and biological assays on *P. viticola* population and isolates of asexual spores (sporangia) were carried out in spring of year 1 (month 8-14) and 2 (month 20-26).

(WP3) Molecular assays on DNA samples collected. Molecular assays to evaluate the point mutations associated to resistance phenotype detected were carried out between month, 10-13, 15-18, 22-28.

(WP4) New method for evaluation of sporangia infection efficiency. The developing of a new method based on flow cytometry and single-cell sorting for isolation of single sporangia was carried out in year 2 (month 13-20).

(WP5) Data analysis. Scientific paper(s) and thesis writing was performed from month 26.

Fungicide Resistance Evolution and Detection in Plant Pathogens: *Plasmopara viticola* as a Case Study

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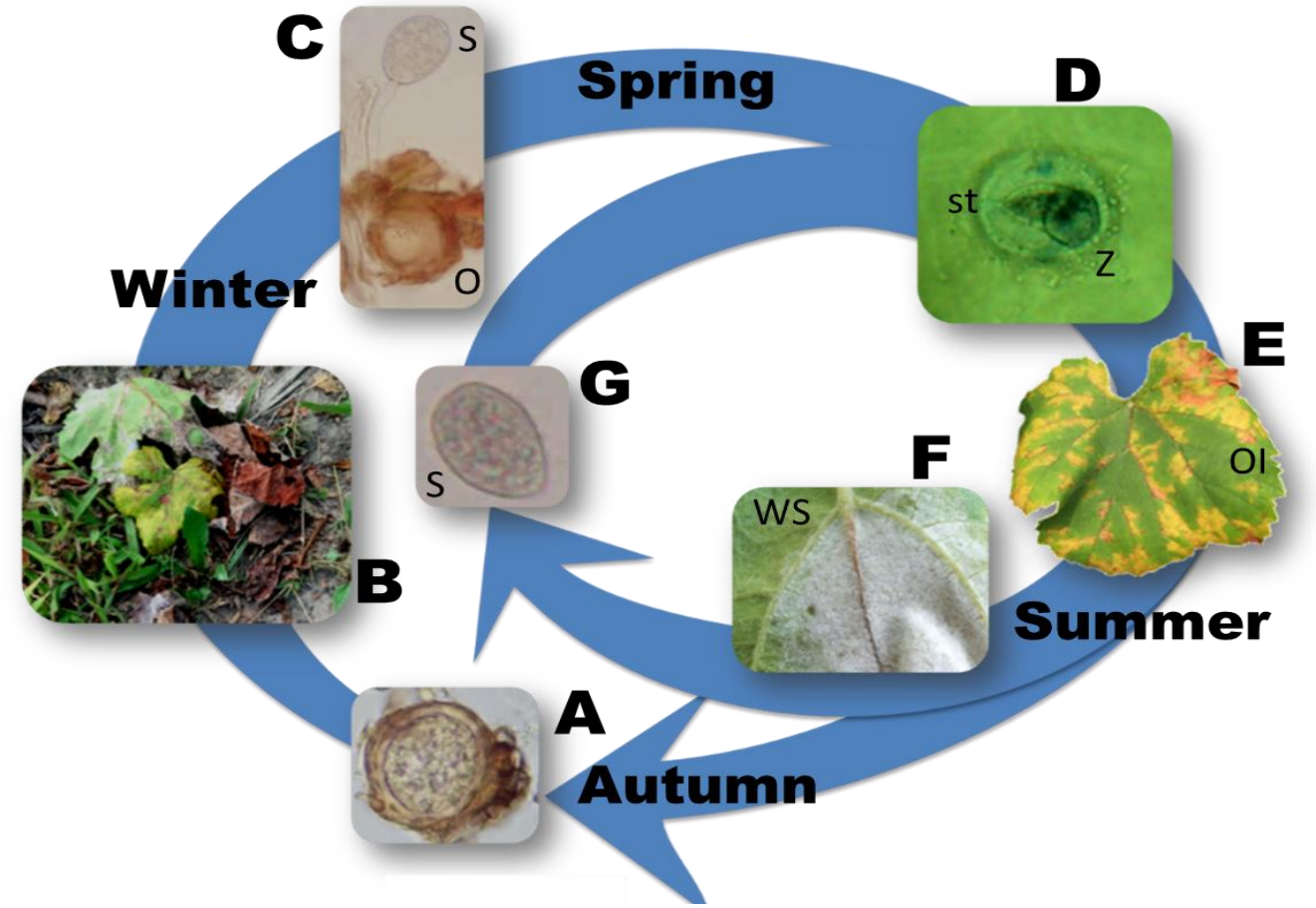
Abstract: The use of single-site fungicides to control plant pathogens in the agroecosystem can be associated with an increased selection of resistant strains. The evolution of resistance represents one of the biggest challenges in disease control. In vineyards, frequent applications of fungicides are carried out every season for multiple years. The agronomic risk of developing fungicide resistance is, therefore, high. *Plasmopara viticola*, the causal agent of grapevine downy mildew, is a high risk pathogen associated with the development of fungicide resistance. *P. viticola* has developed resistance to most of the fungicide classes used and constitutes one of the most important threats for grapevine production. The goals of this review are to describe fungicide resistance evolution in *P. viticola* populations and how to conduct proper monitoring activities. Different methods have been developed for phenotyping and genotyping *P. viticola* for fungicide resistance and the different phases of resistance evolution and life cycles of the pathogen are discussed, to provide a full monitoring toolkit to limit the spread of resistance. A detailed revision of the available tools will help in shaping and harmonizing the monitoring activities between countries and organizations.

1. *Plasmopara viticola*: Characteristics and Management

Downy mildew, caused by the oomycete *Plasmopara viticola*, is one of the major threats for grapevine production, due to the quantitative and qualitative yield losses that are associated with severe disease epidemics (Gessler et al., 2011). *P. viticola* is an obligate parasite of grapevine, causing the main damage to the Eurasian grapevine species (*Vitis vinifera*), which is the most cultivated species worldwide due to the high quality of its grapes. Most of the *V. vinifera* cultivars are highly susceptible to the pathogen, and only recently have sources of resistance been found in the center of origin of viticulture, which is located in Georgia (South Caucasus) (Toffolatti et al., 2018, 2020). This high susceptibility makes chemical control of the pathogen the most important measure to ensure an adequate yield. The timing of fungicide application depends on pathogen features and on weather conditions. *P. viticola* is a polycyclic pathogen, able to undergo numerous infection cycles during a single grapevine growing season. It overwinters as oospores (Figure 1A), which are sexual structures found in dead leaves on the vineyard floor (Figure 1B). In spring, with favorable weather conditions, oospores produce a single macrosporangium (Figure 1C), where the asexual spores (the zoospores) are formed. The zoospores infect the receptive grapevine tissues through stomata (Figure 1D) in the presence of free water, provided by rain or dew, at temperatures below 32 °C. Consequently, frequent fungicide applications are needed in vineyards

located in areas with frequent rainfall and moderate temperatures during the grapevine growing season (Toffolatti et al., 2018).

Figure 1. Disease cycle of *P. viticola*: the pathogen survives the winter period as oospores, i.e., the overwintering structures differentiated by sexual reproduction in autumn **(A)**, embedded in dead leaves on the vineyard floor (**B**). With favorable weather conditions, oospores typically produce sporangia (**C**) that, in turn, produce zoospores (**D**). Zoospores are splashed by rain onto leaves and other receptive tissues of the grapevines, originating the primary infections through stomata penetration (**D**). Disease symptoms, visible as yellow discoloration (oil spots, OI) on the upper side of the leaves (**E**), appear at the end of the incubation period and are followed, in high humidity conditions, by the emission of sporangiophores (**F**) with sporangia (**G**) that will cause secondary infections through the emission of new zoospores. O = oospore; S = sporangium; st = stoma; Z = zoospore; OI = oil spot symptom on the upper side of the leaf; WS = white sporulation, consisting of sporangiophores and sporangia, on the underside of the leaf.



2. The History of the Chemical Control of *P. viticola*

From the end of the Nineteenth Century, when the first agrochemical compounds were tested against *P. viticola*, until now, the panorama of phytoiatric practices has changed greatly, especially because of the availability of new active substances. Although agronomic practices represent a useful tool for disease management and the development of resistant varieties has made great progress, the use of chemical products still represents today the only effective means to control this fungal disease (Jackson, 2008). The growing of traditional varieties of *Vitis vinifera* is not conceivable without the use of fungicide applications (Pertot et al., 2017). The first documented attempts to control downy mildew using chemicals dates

back to 1882, when the French botanist Pierre-Marie-Alexis Millardet noticed that the grapevine plants cultivated along the roadside did not show *P. viticola* symptoms. In the field, only these plants were treated, with a mush made with copper sulphate and lime, to discourage people from eating the grapes. This observation led to the development of the "Bordeaux mixture" to control downy mildew (Millardet, 1885). Its strong efficacy in inhibiting multiple metabolic processes in the fungal pathogen, together with a robust fastness and persistence, made the Bordeaux mixture quickly popular first in Europe, then in Australia and the USA (Lyon, 1924). Among protectant fungicides, copper still represents the most traditional and used chemical. However, intensive use of copper can cause serious environmental problems such as accumulation in the soil and adverse negative effects on beneficial organisms.

The use of the Bordeaux mixture in agriculture was greatly reduced during the Second World War, because copper was preferentially needed by the weapon industries (Liddell Hart, 1970), and its availability for agriculture became secondary. Alternative control compounds were evaluated, but the results were always disappointing (Mestbes, 1942). Experiments were conducted using zinc, aluminum, magnesium sulphates, and other metal salts, such as iron, silver, cadmium, and chromium. After several years of testing, the conclusion was that there were no better alternatives to the Bordeaux mixture (Raucourt, 1943). Because of the scarcity of copper and the absence of options, growers started preparing the Bordeaux mixture with a lower concentration of copper sulphate. Despite the lower dose, disease control was still acceptable in many cases, if the fungicide was employed at the right time during the epidemics. This highlighted the importance of correct and timely applications (Peyer, 1942).

After the Second World War, the first organic fungicides were synthesized by the chemical industry to control downy mildew. The dithiocarbamates and phthalimides were the first chemical classes employed against *P. viticola*. Members of these classes (e.g., zineb and captan), showed similar or higher control than the Bordeaux mixture (Boubals & Vergnes, 1953; Gaudineau & Messiaen, 1953). The success of these fungicides was mainly caused by the higher return on investment and the absence of phytotoxicity, the latter often observed when using copper compounds (Kundert, 1956; Zorbist, 1954). However, intensive use of dithiocarbamates induced an excessive vegetative growth, favoring infections by other pathogens such as *Botrytis cinerea*, the grey mold agent (Jackson, 2008; Pertot et al., 2017; Goshman, 1985; Ye et al., 2002). Environmental toxicity and interference with natural competitors of spider mites like *Tetranychus urticae* and *Panonychus ulmi* (Lorenzon et al., 2018; Posenato, 1994) were reported as well.

A second wave in the development of control solutions occurred between the 1970s and the 1980s, when target-site fungicides were introduced into the market. Target-site fungicides inhibit a single biochemical pathway within the fungal cell (Finch et al., 2014) and generally have a more favorable toxicological profile compared to previous, multisite solutions, which interfere with numerous metabolic processes of the fungus (Edwards et al., 1991; Hawkins & Fraaije, 2018; Rouabhi, 2010). Many of the newly discovered fungicide classes were systemic or cytotropic, i.e., able to penetrate and redistribute in the plant tissues, ensuring a better rain fastness and curative activity (Boubals & Lafon, 1981). The substantial difference between systemic and cytotropic active ingredients is that the former can translocate inside the tissues of the plant (mainly through xylem vessels) and protect the newly formed vegetation, whereas the latter redistribute only locally (Rouabhi, 2010).

3. Fungicide Resistance: A Threat to Downy Mildew Control

With the introduction of target-site fungicides, a new threat soon appeared in downy mildew control: fungicide resistance. Fungicide resistance can be defined as the acquired and heritable reduction in the sensitivity of a fungus to a specific anti-fungal agent (Background Information, www.frac.info). Normally, plant pathogen populations are characterized by a low frequency of resistant individuals that do not interfere with disease control in the open field. Problems with disease control can occur when resistant individuals become predominant over sensitive individuals. The evolution of fungicide resistance in a population is determined by the interaction of different factors, such as the fungicide's mode of action and utilization, the pathogen biology and epidemiology, and the agronomic practices adopted in the field. In the following paragraphs, these factors will be described more in detail and indications on the management of resistance through ad-hoc strategies, aiming at reducing resistance evolution, will be provided, using *P. viticola* and grapevine as a model system.

Fungi and fungal-like organisms such as the oomycetes, where *P. viticola* belongs, share a great capacity of evolution because of their high reproductive frequency (Calo et al., 2013). *P. viticola* is a high risk pathogen because of its complex life cycle, which includes sexual and asexual reproduction and polycyclic behaviors (Figure 1) (Gobbin et al., 2005). The genetic changes that might occur after each reproductive cycle are probably disadvantageous or neutral. However, in some cases, they can provide a fitness advantage (Hawkins & Fraaije, 2018). Fungicide resistance occurs when one of these genetic mutations leads to a stable and heritable reduction in sensitivity to a specific fungicide (FRAC, 2020c). Following repeated treatments with identical active substance, which exerts a selection pressure on the fungal population (Ma & Michailides, 2005), the percentage of sensitive individuals can decrease in favor of resistant mutants. When resistant mutants turn dominant in the population, the pathogen can no longer be adequately controlled by the fungicide (Hewitt, 1998). Fungicides that share the same mode of action should be considered cross-resistant since they inhibit the same target and should not be used without recommendations, thus avoiding the selection of resistant populations (Brent & Hollomon, 2007).

Generally, fungicide resistance can be conferred by five major mechanism: (i) alterations in the target site that decrease binding to the fungicide; (ii) overproduction of the target protein; (iii) presence of an alternative metabolic pathway capable of bypassing the process inhibited by the fungicide; (iv) metabolic breakdown of the fungicide; and (v) active export or exclusion of the fungicide (Brent & Hollomon, 2007; Gullino et al., 2000; McGrath, 2001). The resistance mechanisms known for *P. viticola* can be found in the references listed in Table 1.

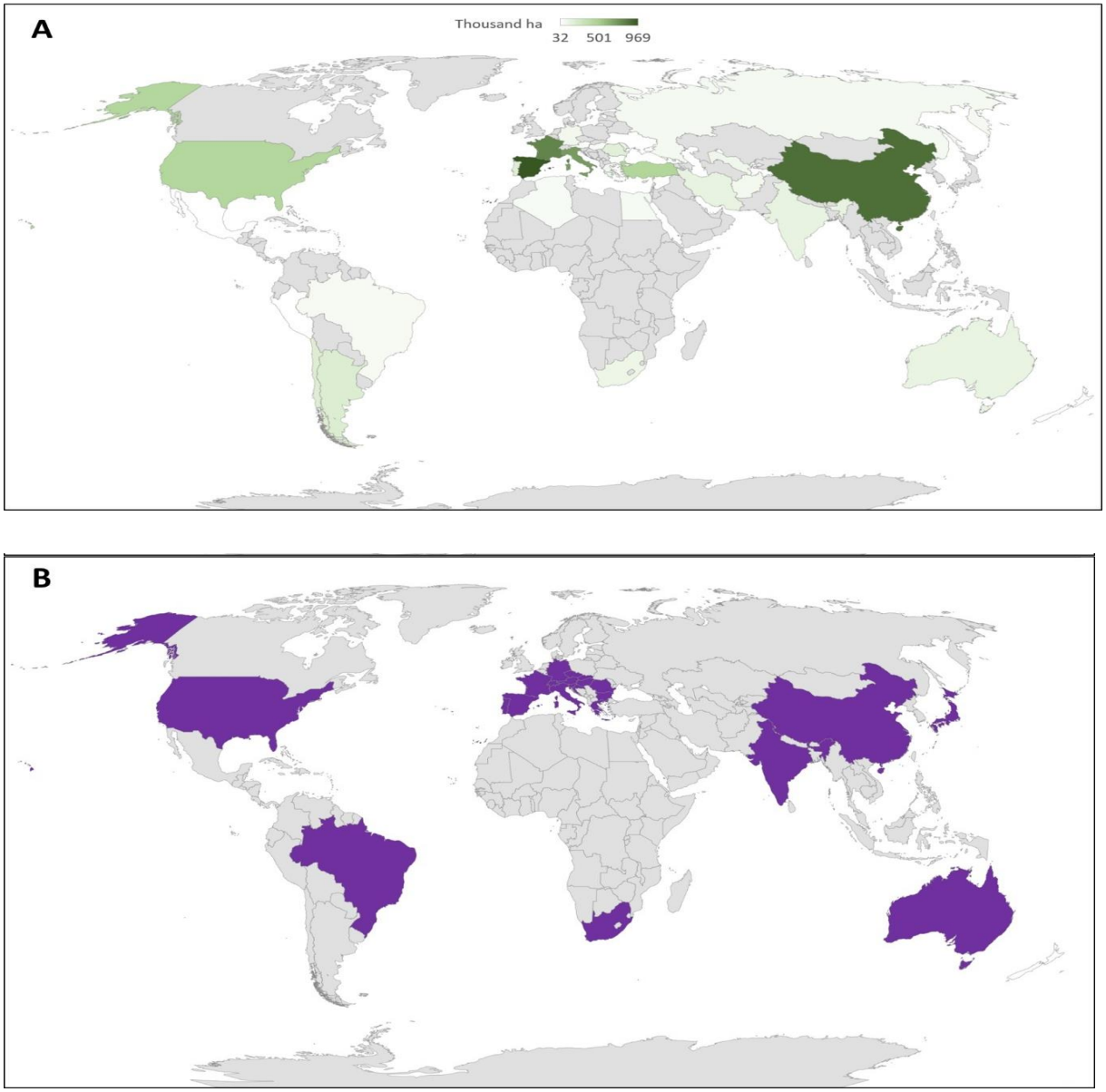
Resistance emerged soon after the introduction of systemic and cytotropic products, from the 1970s onwards (Gisi & Sierotzki, 2008; Hawkins & Fraaije, 2018). The substantial difference between systemic and cytotropic active ingredients is that the former can translocate inside the tissues of the plant (mainly through xylem vessels) and protect the newly formed vegetation, whereas the latter only redistribute locally (Rouabhi, 2010). This happened because, compared to multisite fungicides that interfere with many different metabolic processes, the new molecules were prevalently single-site or site-specific fungicides (Brent & Hollomon, 2007). In the case of targeted fungicides, single nucleotide polymorphisms (SNPs) in the gene encoding for the fungicide target could cause decreased sensitivity. Multisite fungicides, on the other hand, are associated with a lower risk of resistance evolution since several mutations would need to occur simultaneously in different genes in order to prevent the fungicide from binding to its multiple targets (Brent & Hollomon, 2007).

Resistance to different fungicide modes of action in *P. viticola* has been reported (Table 1) in the main vine-growing areas (Figure 2) using different detection techniques (Baudoin et al., 2008; Hall et al., 2017; FRAC, 2020a, 2020b; Furuya et al. 2009; Ghule, et al., 2020; Giraud et al., 2013; Gisi & Sierotzki, 2008; Santos et al., 2020; Wicks & Hall, 2005; Zhang et al., 2017).

Table 1. List of antiperonosporic single/oligo-site active ingredients divided by chemical group, mechanism of action, and resistance reference. "CAA", Carboxylic Acid Amide; "QoI", Quinone outside Inhibitor; "QiI", Quinone inside Inhibitor; "QioI", Quinone inside-outside Inhibitor; "OSBPI", Oxysterol-Binding Protein; "-"not reported

Group Name	Common Name	Chemical Group	Mode of Action	First Confirmed Resistance Reference	
				Report	Remarks
Cyanoacetamide-oxime	Cymoxanil	Cyanoacetamide-oxime	Unknown	Gullino et al., 1997	Reduced field performance
Phenylamides	Metalaxyl, Metalaxyl-M, Benalaxyl, Benalaxyl-M	Acylalanines	Inhibition of ribosomal RNA synthesis	Staub and Sozzi 1981; Bosshard and Schuepp 1983; Leroux and Clerjeau 1985	Reduced field performance
CAA	Dimethomorph	Cinnamic acid amides	Inhibition of cell wall biosynthesis	Gisi et al., 2007	Inheritance of resistance
	Iprovalicarb	Carbamate Vanilamides		Blum et al., 2010	Resistance mechanism
	Bentiavalicarb				
	Valifenalate				
	Mandipropamid	Mandelic acid amides			
QoI	Pyraclostrobin	Strobilurins	Inhibition of mitochondrial respiration, Complex III (Site Qo)	Heaney et al., 2000; Gullino et al., 2004	Reduced field performance
	Famoxadone	Oxazolidinone			
	Fenamidone	Imidazolones		Sierotzki et al., 2005	Review
QiI	Cyazofamid	Cyanoimidazole	Inhibition of mitochondrial respiration, Complex III (Site Qi)	Cherrad et al., 2018; Fontaine et al., 2019	Resistance mechanism
	Amisulbrom	Sulfonamide			
QioI	Ametoctradin	Triazolopyrimidine	Inhibition of mitochondrial respiration, Complex III (Sites Qi and Qo)	Mounkoro et al., 2018, Fontaine et al., 2019	Resistance mechanism
Benzamides	Zoxamide	Toluamides	Inhibition of cellular division	-	-
	Fluopicolide	Pyridinylmethylbenzamides	Delocalizes spectrin-like proteins	Note commune vigne 2020	Unknown mechanism
OSBPI	Oxathiapiprolin	Piperidinyl thianzole isoxazoline	Inhibition of oxysterol binding protein	-	-

Figure 2. Global vine-growing areas allocated for the production of wine grapes, table grapes, or dried grapes in 2018 (sources Organization of Vine and Wine and food and Agriculture Organization of the United Nations) (**A**), compared to countries where *P. viticola* fungicide resistance was reported in 2020 (**B**) (Baudoin et al., 2008; Hall et al., 2017; FRAC, 2020a, 2020b; Furuya et al., 2009; Ghule et al., 2020; Giraud et al., 2013; Gisi & Sierotzki, 2008; Santos et al., 2020; Wicks & Hall, 2005; Zhang et al., 2017).



4. Fungicide Resistance Management

The definition of a balanced fungicide strategy accounting for good disease control and preventing resistance progress is the current challenge. The repeated use of solo fungicides with a single-site mode of action is often associated with a higher risk of resistance evolution when compared to a more diversified approach, e.g., multiple fungicide classes in mixtures or in alternation (Bosch et al., 2014). Anti-resistance strategies are valued in sustainable agriculture since they aim to control the disease and reduce the selection of fungicide resistance. The target of fungicide resistance management is to decrease the selection and diffusion of resistant genotypes in natural populations, as described by the reduction of the selection coefficient (Bosch et al., 2014; Milgroom & Fry, 1988). This value is determined by the combination of the selective

advantage of the resistant strains in the presence of the fungicide and the potential fitness cost associated with resistance in the absence of selection (i.e., negative selection, associated with decreased fitness). Fitness is measured by the per capita rate of increase of the resistant and sensitive strains of a population (Bosch et al., 2014; Hawkins & Fraaije, 2018). The goal of practical management is the reduction of the selection coefficient (i.e., the selection pressure), thus maintaining an acceptable level of disease control and avoiding yield losses (Corio-Costet, 2011).

Grapevine is a perennial plant with a life expectancy of decades; it is cultivated in monoculture, with a period of susceptibility to *P. viticola* of several months that varies each year. It is clear how delicate the management of fungicide resistance for this crop is. The agronomic risk of selecting for resistance associated with vineyards is high, because numerous fungicide sprays are needed every season (Damicone, 2017).

Anti-resistance recommendations can be summarized as follows: use of fungicide mixtures belonging to different classes; avoidance of curative and eradicative applications since they do not allow an adequate control of the pathogen diffusion, which is guaranteed only by preventive fungicide treatments; limitation of the number of treatments per season; application of the fungicide only when strictly required following the recommended dose (Hollomon, 2015). In the specific case of grapevine downy mildew, because of the high pathogenic and agronomic risks, the implementation of correct anti-resistance strategies is challenging (Marina, 2017) and must consider local variations in fungicide sensitivity. The generation of local recommendations, based on specific population sensitivity profiles, requires conducting the organization and carrying out of effective and validated monitoring programs and allow the best application timing in relation to pathogen development (Corio-Costet, 2011).

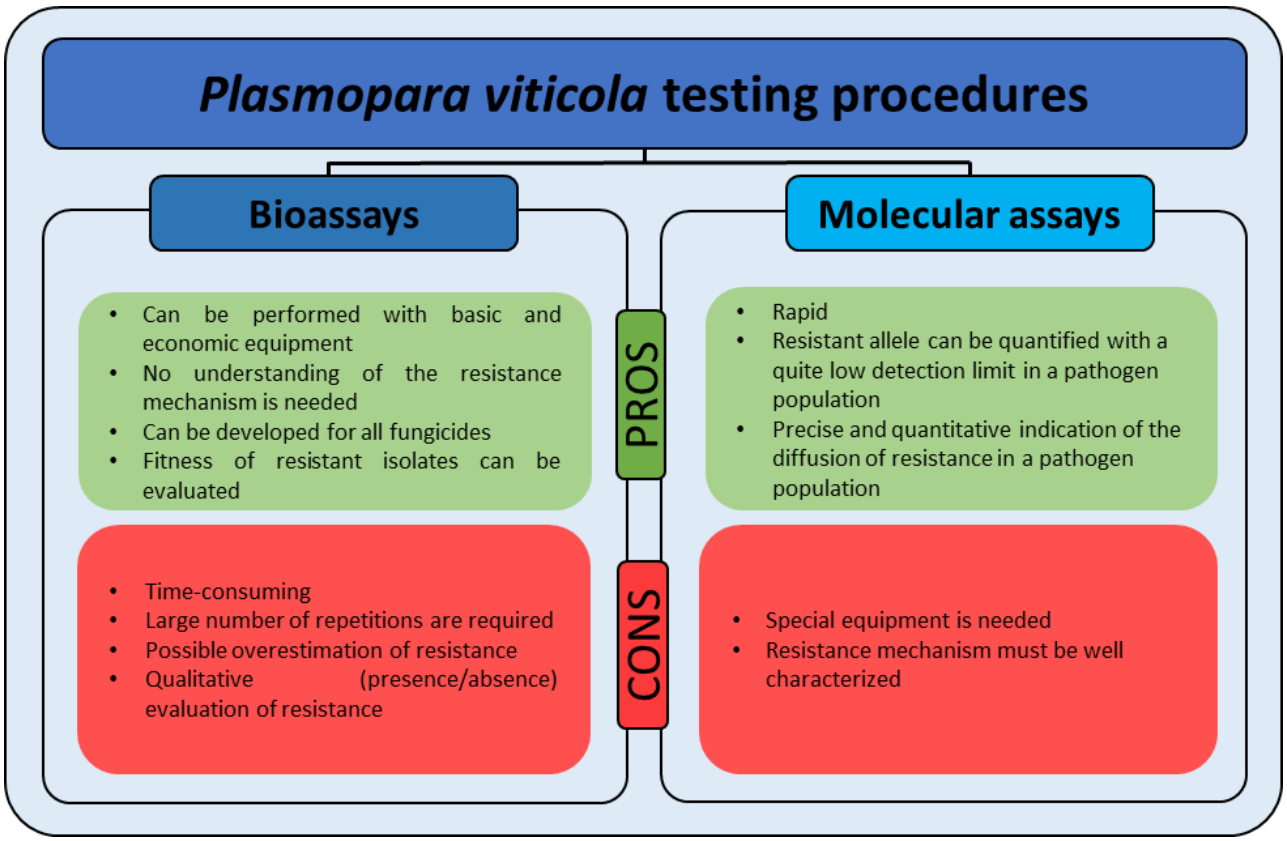
Resistance spread has practical consequences when the lower sensitivity of the pathogen to a fungicide leads to the reduction or loss of disease control in the field (practical resistance). In the worst case, resistance emergence can lead to usage restriction or even suspension of those fungicides with high resistance risk (Gullino, 1987; Hahn, 2014; Collina, 2017). Fungicide resistance reports annually published by FRAC (Fungicide Resistance Action Committee) must be, therefore, carefully interpreted and the recommendations followed in order to avoid practical resistance issues. Still, small changes in sensitivity to fungicides or the rate of resistant individuals at a low frequency have sometimes been overestimated (Brent & Hollomon, 2007). The confirmed presence of a strain showing decreased sensitivity to a fungicide is not necessarily linked to a reduced control of the disease in vineyards. Studies conducted in the laboratory on *P. viticola* sporangia isolates and artificial mixed sporangia populations demonstrated that, in some cases, similar conclusions on fungicide resistance could be drawn with sporangia suspensions containing 1% or 100% resistant sporangia (Genet & Jaworska, 2013). Furthermore, identical *P. viticola* populations tested with different methodologies can generate different results. On the other hand, failures in detecting resistance can be attributed to the choice of methods that are inefficient at quantifying low rates of resistant phenotypes (Corio-Costet, 2015). To limit such false positive and negative issues, the development of standardized, quantitative, reproducible, and readily understandable testing methods has been a primary goal of several organizations such as EPPO (European and Mediterranean Plant Protection Organization) and FRAC. Still, the proper evaluation of the pros and cons of different proposed methods needs years of validation, and not all procedures have the same power when scoring fungicide resistance to different modes of action (Russel, 2004).

5. Strategies for Monitoring Fungicide Resistance

The degree of success of anti-resistance strategies is strongly influenced by the timing of the start of the monitoring activity (Brent, 2012). Resistance monitoring allows detecting changes in the sensitivity of a pest population subjected to different disease pressure levels and spray programs, over several years and in different locations (Ishii, 2006). This activity is usually performed at the national or regional levels, but also by technical world-wide associations such as FRAC (Brent & Hollomon, 2007). What often happens is that monitoring tends to start after indications of decreased sensitivity in the field. As a consequence, monitoring data are not obtained early enough to allow any possible action to preserve the efficacy of the affected product. The initial assessment of the natural range of sensitivity of the pathogen towards the fungicide is, on the contrary, necessary for the interpretation of any shift in further monitoring activities (Wong & Wilcox, 2000). In the past, this kind of information was rarely available, but recently, the agrochemical industry has become committed to presenting baseline sensitivity as part of the registration requirements (Hahn, 2014).

As with other organisms, the detection of resistance in a fungal population can be determined from the comparison between base-line data presented in the literature, which define the normal level of sensitivity of a population never exposed to a specific fungicide, and the data obtained from suspected resistant isolates (Brent, 1992; Georgopoulos, 1982; Lucas et al., 2015). The establishment of validated methods is the first crucial step to create a sensitivity baseline to enable comparisons with subsequent sensitivity data. Fungicide resistance is assessed with different methodologies that can be divided into two main categories: bioassays and molecular assays (Figure 3). Bioassays evaluate the response of the pathogen, in terms of growth and sporulation, to the action of the fungicide (Network, 2016). They can be developed for every fungal species with different levels of complexity (from simple growth on a synthetic medium, for cultivable species, to pathogenicity assessment, for uncultivable species) (Georgopoulos, 1982; Hendricks et al., 2017) and performed in laboratories with basic equipment. Bioassays have the advantage that the sensitivity profile is determined independently of the underlying mechanism of resistance. Their main disadvantages are the long time required to obtain results and the type of information provided: these methods often give a qualitative indication (presence/absence) of resistance occurrence, whereas proper anti-resistance strategies require quantitative information (e.g., percentage of resistant over sensitive individuals) on the pathogen population composition. Molecular assays are performed once the SNP(s) in the fungicide target gene, associated with resistance, is known and allow identification and quantification of the mutated alleles in a population, providing a quantitative indication of resistance rates (Helge Sierotzki & Gisi, 2002). An overview of the criteria and methods, from sampling to data interpretation, developed for monitoring fungicide resistance in *P. viticola* populations is reported in the next paragraphs.

Figure 3. Advantages and disadvantages of biological and molecular assays that should be considered when choosing the testing method.



6. Sampling

The first step of monitoring is field sampling. Two different sampling methods can be applied based on plant development or geography (Brent, 2012). The two approaches are complementary: the first one gives an overall view of resistance at specific plant developmental stages, while the second one evaluates the spread of resistance in vineyards given different disease and treatments' pressures (Parnell et al., 2006). Usually, *P. viticola* samplings are performed at a single stage, after the final fungicide spraying, between August and September. An alternative strategy consisting of multiple collection times, from the beginning to the end of the season, can be very useful for investigating the fitness of *P. viticola* resistant strains and the effects of specific treatments on the selection of the resistant sub-population (Corio-Costet, 2015; Toffolatti et al., 2007; 2011).

At least 50 grapevine leaves with downy mildew symptoms are randomly collected from the vineyard or from specific vineyard plots. Immediately after harvesting, and until arrival in the laboratory, the leaves are preserved in cold conditions to avoid the degradation of the inoculum (Toffolatti et al., 2007; 2018; Corio-costet, 2015; Sierotzki et al., 2005). A critical success factor is related to the proper storage of the samples between collection and testing. It is very difficult to successfully store *P. viticola* on dried plant material; therefore, freezing the material for conservation could be considered. In this case, however, additional investigations with proper controls are needed to test whether or not the viability of the sample has been negatively affected (Russel, 2004).

7. Bioassays

A range of bioassay methods for monitoring fungicide resistance in *P. viticola* have been developed (NetworkR , 2016; Anon, 1991; FRAC 2020d). Since *P. viticola* is an obligate pathogen, it cannot be cultivated or propagated on synthetic media. As a consequence, the use of one of the most common bioassays employed for measuring fungicide sensitivity, the in vitro mycelium growth test on agarized media amended with fungicide, is not possible (Georgopoulos, 1982; Hendrick, 2017; Beckerman, 2013). The most reliable approach to test obligate biotrophs is by experimentally inoculating the pathogen inoculum on entire plants (in planta assays) or detached leaves (in vitro assay) preventively treated with the fungicide of interest (De Miccolis Angelini et al., 2015). Sensitivity is usually measured by determining a toxicological parameter, the EC_{50}, which represents the concentration of fungicide able to inhibit pathogen infection (estimated from the symptomatic area or the area covered by sporulation) by 50% compared to a negative control. By comparing the EC_{50} values of the monitored samples to those present in the baseline, it is possible to quantify shifting in sensitivity (Brent & Hollomon, 2007). Monitoring the fungicide sensitivity of *P. viticola* through bioassays is time-consuming, as it requires sampling, isolation (facultative), and inoculation of the pathogen on living plant material (Fontaine et al., 2019; Wong & Wilcox, 2000). This protocol involves a large number of repetitions to reduce the variability linked to the fact that different leaves can have a different interaction with the pathogen and requires a large production of plant material. Since the isolation of *P. viticola* is difficult and time-consuming, often bulks of strains are tested. This can lead to qualitative results, which tend to overestimate the resistance phenomenon because of the necessary use of high concentrations of spores in the process of artificial inoculation compared to field conditions (Collina, 2017).

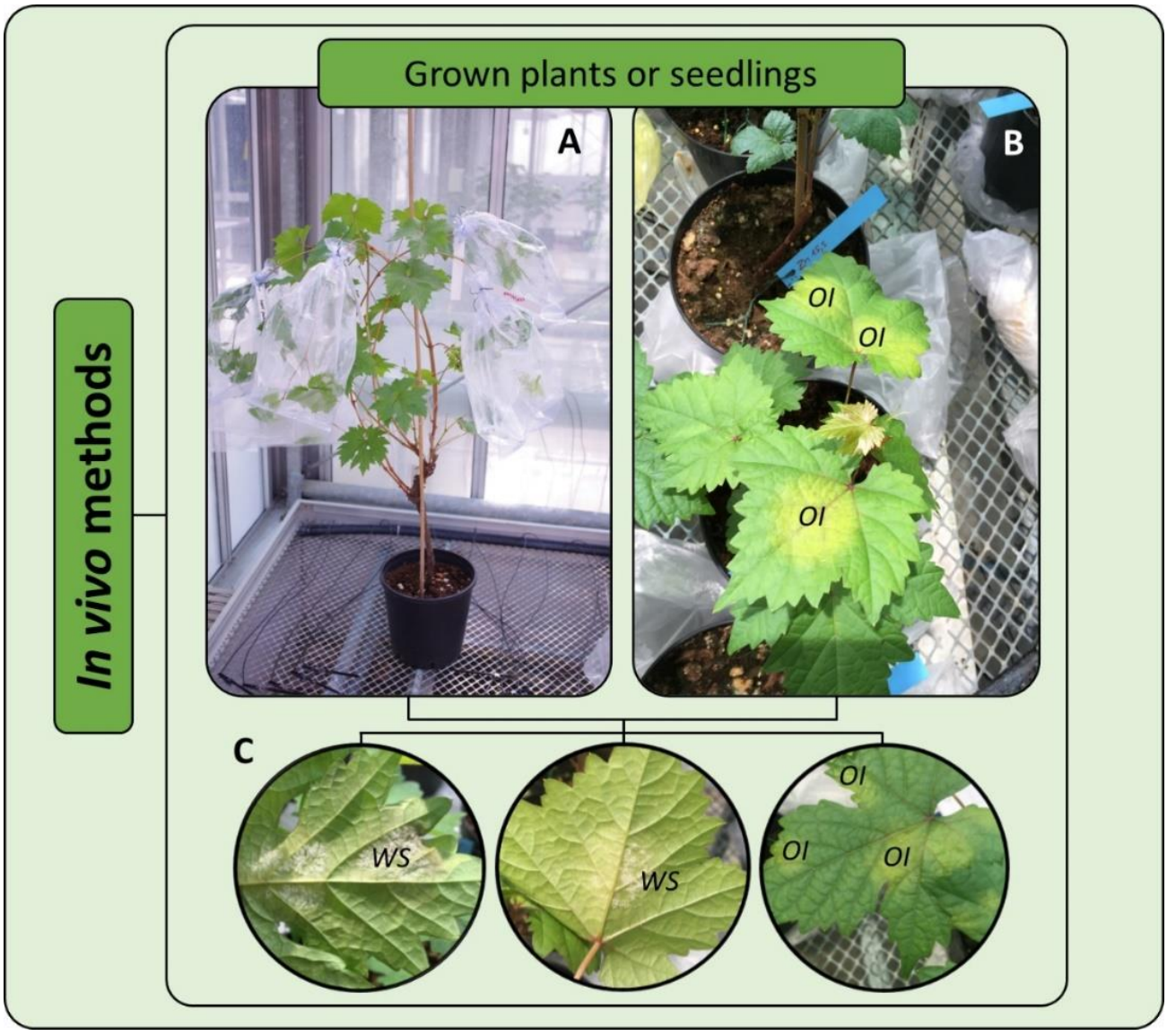
The use of standardized methods and shared reference strains is essential to enable comparisons between different monitoring programs and labs. To achieve this purpose, FRAC published a catalogue of approved standardized methods sorted by pathogen and assay type that allow a direct comparison between results obtained at different research centers (FRAC, 2021). Here, we review a range of methods available to monitor fungicide resistance in *P. viticola* populations in relation to the different resistance evolutionary phases and life cycle of the pathogen. The choice of the test protocol should consider which fungicide, resistance evolutionary phases, and life cycle steps of the pathogen are under investigation. The different methodologies available in the literature to monitor *P. viticola* resistance are described below. Despite the great number of published methods, many of them have been grouped together because of their similarity.

7.1. In Vivo Assays

In the case of *P. viticola*, as for other obligate biotrophs, in vivo tests that are carried out on adult plants or seedlings are challenging. A first issue is related to plant material production during the whole monitoring period that might require a significant logistic effort. The cost and time associated with plant production might be the limiting factors for a high-throughput experiment and can impact the possibility of including replicates, as is normally done for in vitro testing. Whole plant assays are based on the evaluation of pathogenicity on intact plants. The tested fungicide is applied at increasing rates to the leaves (usually the third–fifth from the apex of the shoot) using a laboratory sprayer. The fungicide must be uniformly applied to both the upper and lower side of the leaf one day prior to the inoculation of the sporangia suspension (5×10^4 sporangia/mL) with a handheld sprayer. Formulated products should be preferred instead of the use of technical active ingredients, which might have issues relating to adherence to the plant surface.

Inoculated plants are subsequently incubated in a climate chamber at 20 °C and saturating humidity (Figure 4A) for a period of six days, after which disease assessment is visually performed on three leaves per plant (four plants for treatment as biological or technical replicates) to compare the disease severity of the treated and untreated control samples (Figure 4B,C) (Genet et al., 1997). In some cases, the same population tested using whole plant or detached leaf disc assays can generate different results (Genet & Jaworska, 2013). It appears that changes in physiological and molecular states caused by leaf detachment can contribute to decreasing the host resistance response compared to that of intact plants (Fröbel & Zyprian, 2019; Howard et al., 2000). Furthermore, it may be possible that whole plant assays are ineffective to detect a low proportion of resistant phenotypes (Corio-Costet, 2015).

Figure 4. In vivo tests carried out on grapevine plants (**A**,**B**) aiming at assessing fungicide resistance through the evaluable 50 value of the *P. viticola* population. OI = oil spot symptom on the upper side of the leaf; WS = white sporulation, consisting of sporangiophores and sporangia, on the underside of the leaves.



7.2. In Vitro Assays

In vitro testing for obligate pathogens such as *P. viticola* are based on leaf disc inoculation or on spore germination assessment. The use of those techniques requires a great deal of organization, and for obligate pathogens, this test is usually performed in microtiter plates (Russel, 2004).

7.2.1. Leaf Disc Assay

Many established in vitro tests based on plant tissues are available for *P. viticola* (Clerjeau, 1982; Gullino et al., 2004; Herzog & Schüepp, 1985; Jaworska et al., 2017; Magnien et al., 2012; Reuveni, 2003; Sierotzki, 2014). These methodologies are slightly different, such as for the size of the leaf discs and the way fungicide and inoculum are applied, but all of them allow the testing of large numbers of samples in a short time, using a miniaturized test where portions of the leaves are inoculated with the pathogen. The use of such methods has the great advantage of minimizing the costs in terms of time and resources compared to whole plant assays, and the tests are compatible with all fungicide classes, but in absolute terms, these tests remain highly resource-demanding (Brent & Hollomon, 2007). Moreover, this type of method does not allow a precise evaluation of the percentage of resistant strains in the population tested, since the information they can provide is limited to a qualitative description of the resistance status.

Within this group, two of the approved standard methodologies by FRAC are included: the PLASVI microtiter plate test and PLASVI monitoring (Jaworska et al, 2017; Sierotzki and Kraus, 2014). Considering that *P. viticola* is an obligate biotroph and that assays are often not performed directly on the collected samples, the former method implies propagation of the pathogen on fresh plant material. Collected sporangia are inoculated on new healthy grape leaves placed into a Petri dish containing filter paper soaked with water to prevent dehydration. The Petri dishes are then incubated at 19 °C with a 12h:12h photoperiod inside a plastic box containing soaked filter paper. Fresh sporangia are collected after seven days and resuspended in water, obtaining a sporangia suspension that will be sprayed onto the lower side of fresh healthy leaves using an atomizer. Sporangia suspensions should be standardized at a concentration of 5×10^4 sporangia/mL and applied to 24 leaf discs of 15 mm in diameter placed in a 24-well plate and sprayed with fungicide 24 h before the inoculation (Figure 5A). The discs are incubated in a climate chamber for a period of six days, after which the assessment is visually done by determining the percentage of infected leaf area (Sierotzki & Kraus, 2014). Normally, a range of fungicide concentrations is used in the test to generate an EC_{50} value. An alternative strategy consists of choosing a few discriminatory doses (i.e., doses of fungicides able to discriminate resistance from sensitivity) previously identified as relevant to describe a phenotype. Discriminatory doses are highly effective in the case of a disruptive resistance mechanism such as that associated with SNPs at the target gene of the fungicide. The characterization of EC_{50} is required for fungicides associated with quantitative or semi-quantitative resistance mechanisms.

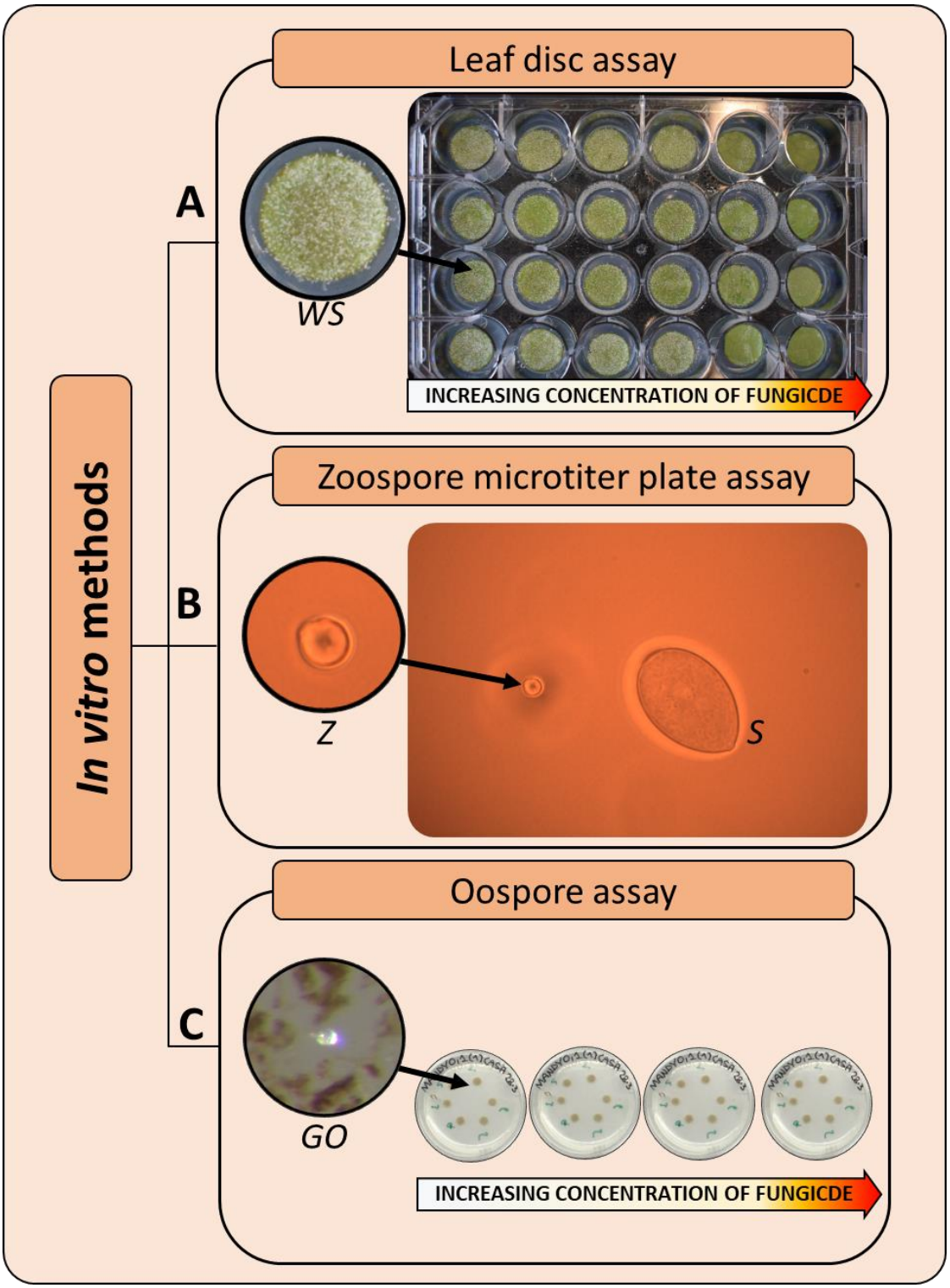
7.2.2. Zoospore Microtiter Plate Assay

Microtiter testing procedures are based on the direct incubation of a sporangia suspension added to increasing concentrations of a fungicide. Such procedures are useful to investigate the inhibitory capacity of a fungicide on zoospores' release and mobility, as in the case of the QoI compound famoxadone (Genet and Vincent, 1999). Fresh sporangia (final density 2.5×10^5 sporangia/mL) harvested in cold water are added into a 96-well microtiter plate containing an aqueous suspension of fungicide at increasing concentrations. Quantification of sporangia germination is visually estimated by observing under microscope the release of zoospores 24 h after incubation at 20 °C in the dark (Figure 5B) and comparing the percentages calculated against those of the negative control (Andrieu et al., 2001; Blum et al., 2010; Genet and Vincent, 1999). However, the reliability of this method is limited since it does not consider a possible osmotic influence on sporangia germination caused by the direct addition of fungicides to the sporangia suspension.

7.2.3. Oospore Assay

Bioassays on *P. viticola* oospores, the sexual and only overwintering structures of the pathogen, can be used to monitor resistance to all those fungicides affecting the differentiation or germination of these structures. The frequency of mutations conferring resistance to some fungicides can fluctuate during the growing seasons, as in the case of carboxylic acid amides (Toffolatti et al., 2018). This test can give an overview of the fungicide resistance state before the occurrence of primary infections, thus allowing a better understanding of the dynamics in the pathogen population and of the extent of selection pressure applied during the previous growing season. The test on oospores implies the collection of samples by randomly sampling leaves showing mosaic symptoms at the end of the grapevine growth season. Leaf fragments rich in oospores are cut out from leaves and placed inside nylon bags (pore size 100 µm) subjected to overwintering in vineyards or in controlled conditions. Germination assays are generally carried out three–five months after the start of overwintering. Fragments are ground in a glass mortar, then filtered through two nylon filters to separate oospores from leaf material (100 and 45 µm), and finally, resuspended in water. The suspension is inoculated and incubated in the dark at 20 °C on water agar plates (1%) containing increasing concentrations of fungicide (Figure 5C). By scoring the frequency of germinated oospores compared to the untreated control, it is possible to quantify the percentage of resistant individuals at a discriminatory fungicide concentration (quantitative evaluation of resistance) (Toffolatti et al., 2007; 2011; 2015; 2018).

Figure 5. In vitro testing for *P. viticola* based on leaf disc bioassay (**A**), zoospore microtiter plates (**B**), and oospore testing (**C**). (**A**) microtiter plate containing leaf discs showing white sporulation (WS). Columns were treated with increasing concentrations of fungicide. (**B**) Sporangium (S) and free zoospore (Z) in liquid medium. (**C**) Agar plates containing increasing concentrations of fungicides and inoculated with oospore suspensions. The number of germinated oospores (GO) is counted and used to calculate the germination percentages at each concentration and to estimate the EC_{50} values of the population or the percentage of resistant oospores at a discriminatory concentration of fungicide.



8. Molecular Assays

For fungicide classes with established molecular mechanisms of resistance, several molecular techniques can be applied for SNP(s) detection in the target gene. Most of the molecular technologies refer to PCR (polymerase chain reaction) and have the advantage of being more rapid and less expensive than biological assays. Besides pure detection, a resistant allele can be quantified with quite a low detection limit in a pathogen population (Sierotzki & Gisi, 2002). The major issue related to molecular monitoring is the need to have a clear understanding of the resistance mechanisms, which is available for only a few fungicide classes. As a consequence, only the well-known resistance alleles can be monitored (NetworkR, 2016). Consequently, molecular assays cannot be used to establish a baseline, and the concepts such as baseline and sensitivity shifting are replaced by the frequency distribution of resistant mutants within a fungal population (Russel, 2004).

The frequency of resistant individuals is extremely low during the initial phases of resistance evolution; therefore, molecular testing represents a useful tool to detect fungicide resistance when conventional bioassays are not able to do so (Miao et al., 2016; Sierotzki & Gisi, 2002). Many advanced molecular tools such as denaturated high performance liquid chromatography (DHPLC), PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), allele specific PCR, allele specific real-time PCR, and droplet digital PCR have been employed with success in the molecular detection of fungicide resistance for different plant pathogens for many years (Ma & Michailides, 2005; Selvaraj et al., 2019). However, the mode of action of the fungicide, the relative resistance mechanism, and the SNPs associated with resistance (Network, 2016) have to be known to run these testing procedures. In the specific case of *P. viticola*, these tests are at present available only for monitoring resistance to quinone outside inhibitors (QoIs) (Chen et al., 2007; Corio-Costet et al., 2011; Gisi et al., 2002), carboxylic acid amides (CAAs) (Toffolatti et al., 2018; Gisi et al., 200; Blum et al., 2010), and more recently, for quinone inside inhibitors (QiIs) and for quinone inside-outside inhibitors (QioIs) (Fontaine et al., 2019; Cherrad et al., 2018; Mounkoro et al., 2019). For other fungicide classes, the mechanisms of resistance are unknown or can potentially involve several genes, greatly complicating the development of molecular tools.

For QoIs and CAAs, resistance mechanisms in *P. viticola* are thoroughly documented (Blum et al., 2012; Grasso et al., 2006; Sierotzki et al., 2007). This has made possible the development of a range of molecular methods. The resistance mechanism to QoI is due to SNPs in the cytochrome b gene (Gisi et al., 2002; Chen et al., 2007; Brasseru et al., 1996). The mutations associated with a shift in sensitivity reported so far are F129L, G137R, and G143A (Sierotzki et al., 2007). Currently, in *P. viticola* isolates, the resistance traits are associated only with F129L or G143A (Delmas et al., 2017; Grasso et al., 2006). The percentage of individuals carrying F129L is significantly lower than the percentage of G143A, which is more widespread and is associated with a particularly high resistance factor (Gisi et al., 2002; S. Toffolatti & Vercesi, 2011). As regards CAAs, a decrease of sensitivity to the fungicide is associated with several SNPs in the third gene of the cellulose synthase complex (*CesA3*). The resistance locus is present in codon 1105 of the *PvCesA3* gene of *P. viticola* and is characterized by a substitution of a glycine (G1105, codon CGC) with a different amino acid (Blum et al., 2012). In European *P. viticola* populations, two possible allelic variants have been detected: the first involves the substitution of glycine with serine (G1105S, codon AGC) and the second one of glycine with valine (G1105V, codon GTG) at position 1105 in the deduced amino acid sequence (Blum et al., 2012; Sierotzki et al., 2011). G1105V is more rarely reported, and most of the time, it is the G1105S mutation that confers resistance to CAAs (Toffolatti et al., 2018).

Rapid molecular testing procedures, aiming at detecting resistance to QoIs and CAAs, have been developed by using PCR-restriction fragment length polymorphism (RFLP) assays (Aoki et al.,

2011; Furuya et al., 2009) and real-time PCR assays (Schwarz et al., 2004; Sierotzki et al., 2005). Compared to the time-consuming bioassays cited above, these PCR based assays can process a large number of *P. viticola* samples simultaneously and quickly became a common tool for the detection and evaluation of fungicide resistance for these two fungicide classes in *P. viticola* isolates. It must be pointed out that PCR-RFLP testing procedures have some intrinsic disadvantages, as an additional restriction enzyme digestion step after PCR amplification is required. To optimize the analytical time and to improve accuracy, the amplification-refractory mutation system PCR assay (ARMS) was developed to detect simultaneously the presence of CAAs and QoI resistant alleles in *P. viticola* populations (Aoki et al., 2013). With this method, the time for detection of mutations is reduced, because no restriction enzyme digestion is required. Unfortunately, this simple and rapid method for the simultaneous detection of *P. viticola* isolates resistant to QoIs and CAAs has some limitations because it can only detect the presence of the resistant alleles and is not able to distinguish between homozygous and heterozygous strains (Zhang et al., 2017). Due to the diploid nature of *P. viticola*, mutations in the coding sequence of genes do not necessarily cause mutant phenotypes. In the case of *PvCesA3*, the resistant G1105S/V character mentioned above is recessive, and it occurs twice in homozygous individuals (−/−) or once in heterozygous ones (−/+) (Blum et al., 2012; Blumer, et al., 2010). For this reason, the use of two parallel PCR assays is required to discriminate between sensitive (−/+) and resistant (−/−) CAA isolates, doubling the workload. To overcome this issue and detect the presence of CAA-resistant strains of *P. viticola* in a single PCR reaction step, a tetra-primer PCR assay (ARMS) was applied to discriminate between homozygous and heterozygous strains (Zhang et al., 2017). In this PCR method, two pairs of primers are present in a single reaction that generates amplicons of different sizes, which allow one to distinguish the presence of two alleles in a single vial: one primer pair is specific for the mutation, and the other one consists of outer primers necessary to create a control band. However, the employment of two sets of primers in one reaction might in some cases lead to cross-amplification and false positives (Hamajima et al., 2002; Huang et al., 2020). To solve this problem and to enhance specificity, sensitivity, and throughput in the detection of resistant and sensitive genotypes, a TaqMan-minor groove binding (MGB)-real time PCR was developed as a more decisive and precise tool (Huang et al., 2020).

9. Conclusions

The use of single-site fungicides for downy mildew control is closely related to the risk of the emergence of resistance. So far, *P. viticola* shows resistance to almost all fungicide classes. Monitoring represents the cornerstone of good resistance management, and the density and magnitude of collected data provide fundamental information about the risk of resistance emergence and spreading. Samples collected on a large scale, in commercial vineyards of different regions or in field trials where the application of the considered fungicide is repeated, could contribute to giving a global and unified vision of the resistance status. The sharing of monitoring results and the communication between public and industrial sectors have key roles in data interpretation and the formulation of recommendations for a sustainable and rational use of the products. The adaptation of *P. viticola* populations to the various selection pressures exerted in the vineyard by the different fungicide classes can be better understood with constant resistance monitoring through several years after resistance emergence in the field.

There is a great diversity among the testing procedures available for monitoring, and different information about the emergence and extension of resistance can be obtained using different methodologies. In the absence of molecular tools, biological tests remain fundamental in monitoring, and the degree of variation compared to a baseline sensitivity represents a good marker of changes in resistance. Considering the various resistance evolutionary phases and the complex life cycle of *P. viticola*, the information on the resistance phenomenon obtained with a

single testing method is not sufficient. The mode of action of the fungicide under investigation, the characteristics of the targeted genetics, and the percentage of resistant strains in the investigated population can strongly influence the results, and the use of multiple testing procedures can help by providing a global and realistic view of resistance evolution.

Bibliography

1. Gessler, C.; Pertot, I.; Perazzolli, M. *Plasmopara viticola*: A review of knowledge on downy mildew of grapevine and effective disease management. *Phytopathol. Mediterr.* 2011, *50*, 3–44.
2. Toffolatti, S.L.; De Lorenzis, G.; Costa, A.; Maddalena, G.; Passera, A.; Bonza, M.C.; Pindo, M.; Stefani, E.; Cestaro, A.; Casati, P.; et al. Unique resistance traits against downy mildew from the center of origin of grapevine (*Vitis vinifera*). *Sci. Rep.* 2018, *8*, 12523.
3. Toffolatti, S.L.; De Lorenzis, G.; Brilli, M.; Moser, M.; Shariati, V.; Tavakol, E.; Maddalena, G.; Passera, A.; Casati, P.; Pindo, M.; et al. Novel aspects on the interaction between grapevine and *Plasmopara viticola*: Dual-seq analysis highlights gene expression dynamics in the pathogen and the plant during the battle for infection. *Genes (Basel).* 2020, *11*, 261.
4. Toffolatti, S.L.; Russo, G.; Campia, P.; Bianco, P.A.; Borsa, P.; Coatti, M.; Torriani, S.F.; Sierotzki, H. A time-course investigation of resistance to the carboxylic acid amide mandipropamid in field populations of *Plasmopara viticola* treated with anti-resistance strategies. *Pest. Manag. Sci.* 2018, *74*, 2822–2834.
5. Jackson, R.S. Vineyard practice. In *Wine science: Principles and Applications*; Academic Press: Burlington, VT, USA, 2008; pp. 108–238.
6. Pertot, I.; Caffi, T.; Rossi, V.; Mugnai, L.; Hoffmann, C.; Grando, M.S.; Gary, C.; Lafond, D.; Duso, C.; Thiery, D.; et al. A critical review of plant protection tools for reducing pesticide use on grapevine and new perspectives for the implementation of IPM in viticulture. *Crop. Prot.* 2017, *97*, 70–84.
7. Millardet, A. Traitement du mildiou par la melange du sulfate de cuivre et de chaux. *Agric. Prat.* 1885, *49*, 707–710.
8. Lyon, A.V. Problems of the viticultural industry. *Bull. Aust.* 1924, *28*, 56–72.
9. Liddell Hart, B.H. *A History of the Second World War*; Pan Books: London, UK, 1970.
10. Mestbes, J.A. La lucha contra el mildiu. *Agricultura* 1942, *11*, 139–140.
11. Raucourt, M. Vue d'ensemble sur les essais anticryptogamiques de 1942. *Ann. Epiphyt.* 1943, *9*, 163–167.
12. Peyer, E. Die Erfahrungen mit schwach konzentrierter Bordeauxbrühe bei der Mehltaubekampfung in den Reben der deutschen Schweiz im Sommer 1941. *Schweiz. Z. Obs. Weinbau* 1942, *51*, 173–178.
13. Gaudineau, M.; Messiaen, C.M. Mildiou de la Vigne et nouveaux produits de lutte. *Agron* 1953, *4*, 185–208.
14. Boubals, D.; Vergnes, A. Essais de fongicides organiques dans la lutte contre le mildiou de la Vigne. *Prog. Agric. Vitic.* 1953, *139*, 90–97.
15. Zorbist, L. L'efficacité des fongicides organiques en viticulture. In *Proceedings III int. Congress on Phyto-pharmacologie*; 1954; Volume 2, pp. 682–688.
16. Kundert, J. Die Peronospora der rebe und ihre bekämpfung im Jahre 1955. *Schweiz. Z. Obs. Weinbau* 1956, *65*, 135–139.
17. Goshman, L.M. Clinical toxicology of commercial products. *Pharm. Sci.* 1985, *74*, 1139.

18. Ye, L.; Dinkova-Kostova, A.T.; Wade, K.L.; Zhang, Y.; Shapiro, T.A.; Talalay, P. Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: Pharmacokinetics of broccoli sprout isothiocyanates in humans. *Clin. Chim. Acta.* 2002, *316*, 43–53.
19. Posenato, G. Popolazioni di *amblyseius aberrans* (Oud.) resistenti ad esteri fosforici e ditiocarbammati. *L'informatore Agrar.* 1994, *24*, 41–43.
20. Lorenzon, M, Pozzebon, A, Duso, C. Biological control of spider mites in North-Italian vineyards using pesticide resistant predatory mites. *Acarologia* 2018, *58*, 98–118.
21. Finch, H.J.S.; Samuel, A.M.; Lane, G.P.F. 6—Diseases of farm crops. In *Lockhart & Wiseman's Crop Husbandry Including Grassland (Ninth Edition)*; Finch, H.J.S., Samuel, A.M., Lane, G.P.F., Eds.; Woodhead Publishing: Sawston, UK, 2014; pp. 119–157.
22. Edwards, R.; Ferry, D.H.G.; Temple, W.A. Fungicides and related compounds. In *Classes of Pesticides*; Hayes, W.J., Laws, E.R., Eds.; Academic Press: San Diego, CA, USA, 1991; pp. 1409–1470.
23. Rouabhi, R. Introduction and toxicology of fungicides. In *Fungicides*; Carisse, O. Ed.; Intech Open: London, UK, 2010.
24. Hawkins, N.J.; Fraaije, B.A. Fitness penalties in the evolution of fungicide resistance. *Annu. Rev. Phytopathol.* 2018, *56*, 339–360.
25. Boubals, D.; Lafon, R. Control of grapevine downy mildew by penetrating systemic fungicides. *Bull. L'organisation Int. Vigne Vin* 1981, *54*, 319–335.
26. Calo, S.; Billmyre, R.B.; Heitman, J. Generators of phenotypic diversity in the evolution of pathogenic microorganisms. *PLoS Pathog.* 2013, *9*, e1003181.
27. Gobbin, D.; Jermini, M.; Loskill, B.; Pertot, I.; Raynal, M.; Gessler, C. Importance of secondary inoculum of *Plasmopara viticola* to epidemics of grapevine downy mildew. *Plant. Pathol.* 2005, *54*, 522–534.
28. FRAC(a) Resistance Overview. Available online: https://www.frac.info/fungicide-resistance-management/background (accessed on 17 November 2020).
29. Ma, Z.; Michailides, T.J. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop. Prot.* 2005, *24*, 853–863.
30. Hewitt, H.G. *Fungicides in Crop Protection*; CAB International: Wallingford, UK, 1998.
31. Brent, K.J.; Hollomon, D.W. Fungicide resistance in crop pathogens. How can it be managed. In *FRAC Monograph*; No. 1.; CropLife International: Brussels, Belgium, 2007.
32. Gullino, M.L.; Leroux, P.; Smith, C.M. Uses and challenges of novel compounds for plant disease control. *Crop. Prot.* 2000, *19*, 1–11.
33. McGrath, M.T. Fungicide resistance in cucurbit powdery mildew: Experiences and challenges. *Plant. Dis.* 2001, *85*, 236–245.
34. Gisi, U.; Sierotzki, H. Fungicide modes of action and resistance in downy mildews. In *The Downy Mildews—Genetics, Molecular Biology and Control*; Lebeda, A., Spencer-Phillips, P.T.N., Cooke, B.M., Eds.; Springer: Dordrecht, The Nederland, 2008; pp. 157–167.
35. Baudoin, A.; Olaya, G.; Delmotte, F.; Colcol, J.F.; Sierotzki, H. QoI Resistance of *Plasmopara viticola* and erysiphe necator in the Mid-Atlantic United States. *Plant. Heal. Prog.* 2008, *9*, 25.

36. Giraud, F.; Molitor, D.; Bleunven, M.; Evers, D. Fungicide sensitivity profiles of the *Plasmopara viticola* populations in the Luxembourgian grape-growing region. *J. Plant. Pathol.* 2013, *1*, 55–62.

37. Hall, B.H.; McKay, S.F.; Lopez, F.; Harper, L.; Savocchia, S.; Borneman, A.; Herderich, M. Fungicide resistance in Australian viticulture. In *Modern Fungicides and Antifungal Compounds*; Deutsche Phytomedizinische Gesellschaft: Braunschweig, Germany, 2017; Volume VIII, pp. 181–186.

38. Santos, R.F.; Fraaije, B.A.; Garrido, L.d.R.; Monteiro-Vitorello, C.B.; Amorim, L. Multiple resistance of *Plasmopara viticola* to QoI and CAA fungicides in Brazil. *Plant. Pathol.* 2020, *69,* 1708–1720.

39. Furuya, S.; Suzuki, S.; Kobayashi, H.; Saito, S.; Takayanagi, T. Rapid method for detecting resistance to a QoI fungicide in *Plasmopara viticola* populations. *Pest. Manag. Sci.* 2009, *65*, 840–843.

40. Ghule, M.R.; Sawant, I.S.; Sawant, S.D.; Saha, S. Resistance of *Plasmopara viticola* to multiple fungicides in vineyards of Maharashtra, India. *J. Env. Biol.* 2020, *41*, 1026–1033.

41. Wicks, T.J.; Hall, B.H. First report of metalaxyl resistance of grapevine downy mildew in Australia. In *The 15th Biennial Australasian Plant Pathology Society Conference Handbook*; Australasian Plant Pathology Society: Geelong, Australia, 2005; p. 319.

42. Zhang, H.; Kong, F.; Wang, X.; Liang, L.; Schoen, C.D.; Feng, J.; Wang, Z. Tetra-primer ARMS PCR for rapid detection and characterisation of *Plasmopara viticola* phenotypes resistant to carboxylic acid amide fungicides. *Pest. Manag. Sci.* 2017, *73*, 1655–1660.

43. FRAC(b) QoI WG Meeting and Recommendations for 2020. Available online: https://www.frac.info/docs/default-source/working-groups/qol-fungicides/qoi-meeting-minutes/minutes-of-the-2020-qoi-wg-meeting-and-recommendations-for-2020-(-june-september-2020-updates).pdf?sfvrsn=21b8499a_20 (accessed on 17 November 2020).

44. FRAC(c) Minutes of the 2020 CAA Meeting Recommendations for 2020. Available online https://www.frac.info/docs/default-source/working-groups/caa-fungicides/caa-wg/minutes-of-the-2020-caa-meeting-recommendations-for-2020.pdf?sfvrsn=6e67499a_2 (accessed on 17 November 2020).

45. Bosch, F.V.D.; Oliver, R.; Berg, F.V.D.; Paveley, N. Governing principles can guide fungicide-resistance management tactics. *Annu. Rev. Phytopathol.* 2014, *52*, 175–195.

46. Milgroom, M.G.; Fry, W.E. A simulation analysis of the epidemiological principles for fungicide resistance management in pathogen populations. *Phytopathology* 1988, *78*, 565–570.

47. Corio-Costet, M.-F. Fungicide resistance in *Plasmopara viticola* in France and anti resistance measures. In *Fungicide Resistance in Crop Protection: Risk and Management*; CABI Publishing: Oxford, UK, 2011; p. 304.

48. Damicone, J. Fungicide Resistance Management; Fact Sheet EPP; Oklahoma Cooperative Extension Service: Oklahoma City, OK, USA, 2017, p. 7663.

49. Hollomon, D.W. Fungicide resistance: Facing the challenge. *Plant. Prot. Sci.* 2015, *51*, 170–176.

50. Collina, M. Resistenza agli antiperonosporici, concetti chiave e prevenzione. *Suppl. A L'informatore Agrar.* 2017, *13*, 8–10.

51. Gullino, M.L. La resistenza ai fungicidi. *Inf. Fitopatol.* 1987, *37*, 8–12.

52. Hahn, M. The rising threat of fungicide resistance in plant pathogenic fungi: *Botrytis* as a case study. *J. Chem. Biol.* 2014, *7*, 133–141.

53. Genet, J.L.; Jaworska, G. Characterization of European *Plasmopara viticola* isolates with reduced sensitivity to cymoxanil. *Eur. J. Plant. Pathol.* 2013, *135*, 383–393.

54. Corio-Costet, M.-F. Monitoring resistance in obligate pathogens by bioassays relating to field use: Grapevine powdery and downy mildews. In *Fungicide Resistance in Plant Pathogens*; Springer: Tokyo, Japan, 2015.

55. Russel, P. Sensitivity baselines in fungicide resistance research and management. In *FRAC Monograph 3*; CropLife International: Brussels, Belgium, 2004.

56. Brent, K.J. Historical perspectives of fungicide resistance. In *Fungicide Resistance in Crop Protection: Risk and Management*; Tarlochan, S., Ed.; CABI: Wallingford, UK, 2012; pp. 3–18.

57. Hideo, I. Impact of fungicide resistance in plant pathogens on crop disease control and agricultural environment. *Jpn. Agric. Res. Q.* 2006, *40*, 205–211.

58. Wong, F.P.; Wilcox, W.F. Distribution of baseline sensitivities to azoxystrobin among isolates of *Plasmopara viticola*. *Plant. Dis.* 2000, *84*, 275–281.

59. Georgopoulos, S.G. Detection and measurement of fungicide-resistance. In *Fungicide-Resistance in Plant Protection*; Dekker, J., Georgopoulos, S.G., Eds.; Pudoc: Wageningen, The Netherlands, 1982; pp. 24–31.

60. Brent, K.J. Monitoring fungicide resistance: Purposes, procedures and progress. In *Resistance '91: Achievements and Developments in Combating Pesticide Resistance*; Denholm, I., Devonshire, A.L., Hollomon, D.W., Eds.; Springer: Dordrecht, The Netherlands, 1992; pp. 1–18.

61. Lucas, J.A.; Hawkins, N.J.; Fraaije, B.A. Chapter two—The evolution of fungicide resistance. In *Advances in Applied Microbiology*; Sariaslani, S., Gadd, G.M., Eds.; Academic Press: Cambridge, MA, USA, 2015; Volume 90, pp. 29–92.

62. Network, R. Trends and challenges in pesticide resistance detection. *Trends Plant Sci.* 2016, *21*, 834–853.

63. Hendricks, K.E.; Christman, M.C.; Roberts, P.D. A statistical evaluation of methods of in-vitro growth assessment for phyllosticta citricarpa: Average colony diameter vs. area. *PLoS ONE* 2017, *12*, 1–7.

64. Sierotzki, H.; Gisi, U. Molecular diagnostics for fungicide resistance in plant pathogens. In *Chemistry of Crop Protection*; John Wiley & Sons, Ltd: Hoboken, NJ, USA, 2002; pp. 71–88.

65. Parnell, S.; Bosch, F.V.D.; Gilligan, C.A. Large-scale fungicide spray heterogeneity and the regional spread of resistant pathogen strains. *Phytopathology* 2006, *96*, 549–555.

66. Toffolatti, S.L.; Serrati, L.; Sierotzki, H.; Gisi, U.; Vercesi, A. Assessment of QoI resistance in *Plasmopara viticola* oospores. *Pest. Manag. Sci.* 2007, *63*, 194–201.

67. Toffolatti, S.; Vercesi, A. QoI resistance in *Plasmopara viticola* in Italy: Evolution and management strategies. In *Fungicide Resistance in Crop Protection: Risk and Management*; Springer: New York, NY, USA, 2011; pp. 172–183.

68. Sierotzki, H.; Kraus, N.; Assemat, P.; Stanger, C.; Cleere, S.; Windass, J. Evolution of resistance to QoI fungicides in *Plasmopara viticola* populations in Europe. In *Modern Fungicides and Antifungal Compounds IV.*; Dehne, H.W., Gisi, U., Kuck, K.H., Russell, P.E., Lyr, H., Eds.; BCPC: Alton, UK, 2005; pp. 73–80.

69. Anon. Methods for monitoring fungicide resistance. *EPPO Bull.* 1991, *20*, 253–260.
70. FRAC(d) List of Monitoring Methods. Available online: https://www.frac.info/knowledge-database/monitoring-methods (accessed on 17 November 2020).
71. Beckerman, J.L. Detection of fungicide resistance. In *Fungicides—Showcases of Integrated Plant Disease Management from Around the World*; Nita, M., Ed.; InTech: London, UK, 2013; pp. 281–310.
72. De Miccolis Angelini, R.M.; Pollastro, S.; Faretra, F. Genetics of fungicide resistance. In *Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management*; Ishii, H., Hollomon, D.W., Eds.; Springer: Tokyo, Japan, 2015; pp. 13–34.
73. Fontaine, S.; Remuson, F.; Caddoux, L.; Barrès, B. Investigation of the sensitivity of *Plasmopara viticola* to amisulbrom and ametoctradin in French vineyards using bioassays and molecular tools. *Pest. Manag. Sci.* 2019, *75*, 2115–2123.
74. Genet, J.L.; Steva, H.; Vincent, O.; Cazenave, C. A method for measuring the level of sensitivity of *Plasmopara viticola* populations to cymoxanil. *EPPO Bull.* 1997, *27*, 217–225.
75. Howard, R.J.; Ferrari, M.A.; Genet, J.L.; Stidham, M. Biology of curzate action against *Plasmopara viticola* infection of grape. In Proceedings of the AFPP-Sixième Conférence Internationale sur les Maladies des Plantes, Tours, France, 2000.
76. Fröbel, S.; Zyprian, E. Colonization of different grapevine tissues by *Plasmopara viticola*—A histological study. *Front. Plant. Sci.* 2019, *10*, 951.
77. Clerjeau, M.S.J. Apparition en France de souches de mildiou (*Plasmopara viticola*) résistantes aux fongicides de la famille des anilides (métalaxyl, milfuram). *Progr. Agric. Vitic.* 1982, *99*, 59–61.
78. Herzog, J.; Schüepp, H. Haustorial development test to characterize metalaxyl resistance and genetic variability in *Plasmopara viticola*. *EPPO Bull.* 1985, *15*, 431–435.
79. Reuveni, M. Activity of the new fungicide benthiavalicarb against *Plasmopara viticola* and its efficacy in controlling downy mildew in grapevines. *Eur. J. Plant. Pathol.* 2003, *109*, 243–251.
80. Gullino, M.L.; Gilardi, G.; Tinivella, F.; Garibaldi, A. Observations on the behaviour of different populations of *Plasmopara viticola* resistant to QoI fungicides in Italian vineyards. *Phytopathol. Mediterr.* 2004, *43*, 341–350.
81. Magnien, C.; Remuson, F.; Guellec, M.L.E.; Micoud, A.; Grosman, J. *Grape Vine Downy Mildew Resistance to Fungicides—Results of the Monitoring Directed by the French Plant Protection Organization Between 2009 and 2011*; Association Française de Protection des Plantes (AFPP): Alfortville, France, 2012; pp. 290–298.
82. Sierotzki, H.; Kraus, N. PLASVI Microtiter. Available online: https://www.frac.info/docs/default-source/monitoring-methods/approved-methods/plasvi-microtiter.pdf?sfvrsn=409a419a_4 (accessed on 17 November 2020).
83. Jaworska, G.; Genet, J.L.; Kane, M. PLASVI Monitoring Method OSBI DuPont. Available online: https://www.frac.info/docs/default-source/monitoring-methods/approved-methods/plasvi-monitoring-method-osbpi-dupont-2017.pdf?sfvrsn=6cae489a_2 (accessed on 17 November 2020).
84. Genet, J.L.; Vincent, O. Sensitivity to Famoxate (famoxadone) of European *Plasmopara viticola* populations. *Agris* 1999, *64*, 559–564.

85. Andrieu, N.; Jaworska, G.; Genet, J.L.; Bompeix, G. Biological mode of action of famoxadone on *Plasmopara viticola* and *Phytophthora infestans*. *Crop. Prot.* 2001, *20*, 253–260.

86. Blum, M.; Boehler, M.; Randall, E.; Young, V.; Csukai, M.; Kraus, S.; Moulin, F.; Scalliet, G.; Avrova, A.O.; Whisson, S.C.; et al. Mandipropamid targets the cellulose synthase-like PiCesA3 to inhibit cell wall biosynthesis in the oomycete plant pathogen, *Phytophthora infestans*. *Mol. Plant. Pathol.* 2010, *11*, 227–243.

87. Toffolatti, S.L.; Venturini, G.; Campia, P.; Cirio, L.; Bellotto, D.; Vercesi, A. Sensitivity to cymoxanil in Italian populations of *Plasmopara viticola* oospores. *Pest. Manag. Sci.* 2015, *71*, 1182—1188.

88. Miao, J.; Dong, X.; Lin, D.; Wang, Q.; Liu, P.; Chen, F.; Du, Y.; Liu, X. Activity of the novel fungicide oxathiapiprolin against plant-pathogenic oomycetes. *Pest. Manag. Sci.* 2016, *72*, 1572–1577.

89. Selvaraj, V.; Maheshwari, Y.; Hajeri, S.; Yokomi, R. Droplet digital PCR for absolute quantification of plant pathogens. In *Plant Biotechnology: Progress in Genomic Era*; Khurana, S.M.P., Gaur, R.K., Eds.; Springer: Singapore, 2019; pp. 583–595.

90. Gisi, U.; Sierotzki, H.; Cook, A.; McCaffery, A. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest. Manag. Sci.* 2002, *58*, 859–867.

91. Chen, W.-J.; Delmotte, F.; Richard-Cervera, S.; Douence, L.; Greif, C.; Corio-Costet, M.-F. At least two origins of fungicide resistance in grapevine downy mildew populations. *Appl. Env. Microbiol.* 2007, *73*, 5162–5172.

92. Corio-Costet, M.-F.; Dufour, M.-C.; Cigna, J.; Abadie, P.; Chen, W.-J. Diversity and fitness of *Plasmopara viticola* isolates resistant to QoI fungicides. *Eur. J. Plant Pathol.* 2011, *129*, 315–329.

93. Gisi, U.; Waldner, M.; Kraus, N.; Dubuis, P.H.; Sierotzki, H. Inheritance of resistance to carboxylic acid amide (CAA) fungicides in *Plasmopara viticola*. *Plant Pathol.* 2007, *56*, 199–208.

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

95. Cherrad, S.; Hernandez, C.; Steva, H.; Vacher, S. Resistance de *Plasmopara viticola* aux inhibiteurs du complexe III: Un point sur la caracterisation phenotypique et genotypique des souches. In 12e Conférence Internationale sur les Maladies des Plantes*,* Tours, France, 11 - 12 December 2018; pp. 449–459.

96. Mounkoro, P.; Michel, T.; Benhachemi, R.; Surpateanu, G.; Iorga, B.I.; Fisher, N.; Meunier, B. Mitochondrial complex III Qi-site inhibitor resistance mutations found in laboratory selected mutants and field isolates. *Pest. Manag. Sci.* 2019, *75*, 2107–2114.

97. Sierotzki, H.; Frey, R.; Wullschleger, J.; Palermo, S.; Karlin, S.; Godwin, J.; Gisi, U. Cytochrome b gene sequence and structure of Pyrenophora teres and P. tritici-repentis and implications for QoI resistance. *Pest. Manag. Sci.* 2007, *63*, 225–233.

98. Blum, M.; Gamper, H.A.; Waldner, M.; Sierotzki, H.; Gisi, U. The cellulose synthase 3 (CesA3) gene of oomycetes: Structure, phylogeny and influence on sensitivity to carboxylic acid amide (CAA) fungicides. *Fungal Biol.* 2012, *116*, 529–542.

99. Grasso, V.; Palermo, S.; Sierotzki, H.; Garibaldi, A.; Gisi, U. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest. Manag. Sci.* 2006, *62*, 465–472.

100. Brasseur, G.; Saribaş, A.S.; Daldal, F. A compilation of mutations located in the cytochrome b subunit of the bacterial and mitochondrial bc1 complex. *Biochim. Biophys. Acta* 1996, *1275*, 61–69.

101. Delmas, C.E.L.; Dussert, Y.; Delière, L.; Couture, C.; Mazet, I.D.; Richart Cervera, S.; Delmotte, F. Soft selective sweeps in fungicide resistance evolution: Recurrent mutations without fitness costs in grapevine downy mildew. *Mol. Ecol.* 2017, *26*, 1936–1951.

102. Sierotzki, H.; Blum, M.; Olaya, G.; Waldner-Zulauf, M.; Buitrago, C.; Wullschleger, J.; Cohen, Y.; Gisi, U. Sensitivity to CAA fungicides and frequency of mutations in cellulose synthase (*CesA3*) gene of oomycete pathogen populations. In Proceedings of the Modern Fungicides and Antifungal Compounds VI. 16th International Reinhardsbrunn Symposium, Friedrichroda, Germany, 25–29 April 2010; Deutsche Phytomedizinische Gesellschaft e.V. Selbstverlag: Brunswick, Germany, 2011; pp. 103–110.

103. Aoki, Y.; Furuya, S.; Suzuki, S. Method for rapid detection of the PvCesA3 gene allele conferring resistance to mandipropamid, a carboxylic acid amide fungicide, in *Plasmopara viticola* populations. *Pest. Manag. Sci.* 2011, *67*, 1557–1561.

104. Schwarz, G.; Bäumler, S.; Block, A.; Felsenstein, F.G.; Wenzel, G. Determination of detection and quantification limits for SNP allele frequency estimation in DNA pools using real time PCR. *Nucleic Acids Res.* 2004, *32*, e24.

105. Aoki, Y.; Hada, Y.; Suzuki, S. Development of a multiplex allele-specific primer PCR assay for simultaneous detection of QoI and CAA fungicide resistance alleles in *Plasmopara viticola* populations. *Pest. Manag. Sci.* 2013, *69*, 268–273.

106. Hamajima, N.; Saito, T.; Matsuo, K.; Tajima, K. Competitive amplification and unspecific amplification in polymerase chain reaction with confronting two-pair primers. *J. Mol. Diagn.* 2002, *4*, 103–107.

107. Huang, X.; Wang, X.; Kong, F.; van der Lee, T.; Wang, Z.; Zhang, H. Detection and characterization of carboxylic acid amide-resistant *Plasmopara viticola* in China using a taqman-mgb real-time PCR. *Plant. Dis.* 2020, *104*, 2338–2345.

Monitoring of sensitivity of *P. viticola* populations exposed to a variable number of treatments with zoxamide in North-western Italy.

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Abstract

Zoxamide is an important fungicide for disease management of many oomycetes, including *Plasmopara viticola*, the causal agent of grapevine downy mildew. In this study, *P. viticola* populations isolated from 50 vineyards located in Northern Italy were tested for their sensitivity to zoxamide. Sensitivity was evaluated by the oospore germinability on zoxamide-amended media at discriminating dose and by the EC_{50} calculation. In general, the populations tested were characterized by good sensitivity levels to zoxamide and the mean EC_{50} value was of 0.022 mg/L. Results of this study will be helpful for the management of fungicide resistance in *P. viticola* chemical control.

Introduction

Zoxamide is a crop protection fungicide for foliar use, which belongs to the B3 FRAC classification (cytoskeleton and motor proteins), in particular to the benzamides sub-group (FRAC, 2021).
Since its introduction in the early 2000's, this active substance has proved to be highly effective to control many diseases caused by fungal-like organisms belonging to the oomycete group, including *Plasmopara viticola* (Berk. *et* Curt) Berlese and de Toni, one of the most devastating diseases of *Vitis vinifera* L. (Ruggiero & Regiroli, 2000). In detail, this fungicide is one of the 16 single/oligo-site fungicides actually available for grapevine downy mildew chemical control (Massi et al., 2021). Zoxamide binds covalently and non-reversely to β-tubulin, avoiding the fastening of α-tubulin, therefore disrupting microtubule formation and finally hijacking nuclear division, as microtubules are key components of the mitotic spindle (Young & Slawecki, 2001).

Given its nature of single-site fungicide, it must be noticed that zoxamide can be potentially involved with the phenomenon of fungicide resistance and a consequent possible reduction of effectiveness (Brent, 2012). Fungicide resistance can be defined as the acquired and heritable reduction in the sensitivity of a fungus to a specific anti-fungal agent (FRAC, 2020), and represents nowadays one of the greatest challenges in downy mildew control. Zoxamide still represents a valid alternative to other fungicides in downy mildew control, primarily because to date no sign of concrete in-field resistance has been found for this pathogen (Massi et al., 2021). However, *P.* viticola possesses a great capacity for evolution and can adapt fast to adverse environmental condition, such as repeated fungicide applications (Calo et al., 2013). The polycyclic nature of this

pathogen enables it to produce large quantities of inoculum per year (Calo et al., 2013), therefore exponentially increasing the chance of resistant individuals (Gessler et al., 2011).

In a laboratory-only trial performed in 2006, Ziogas et al. actually succeeded into selecting various mutant strains of *Phytophthora Infestans* (Mont.) de Bary, a clear example of high-resistance risk oomycete, which displayed tolerance to zoxamide and even in some cases full cross-resistance among non-related fungicides (Ziogas et al., 2006). Although resistance against zoxamide has not been yet discovered in any in-field population of any oomycetes, it cannot be ignored that, in light of this given evidence, genetical bases of mutations in the β-tubulin gene of oomycetes conferring resistance exist (Cai et al., 2016), and pose the threat of preventing the outbreak of more serious practical resistances.

Pending the elucidation of a possible mechanism of resistance of *P. viticola* to zoxamide, and therefore the availability of molecular methods to monitor resistance to this fungicide, careful and precise monitoring with biological assays should be pursued to avoid the sudden onset of *P. viticola* infections eventually no more containable with zoxamide (Corio-Costet, 2015).

Among the biological assays available for monitoring fungicide resistance in *P. viticola* populations, the assays carried out by evaluating the germinability of the sexual spores of the pathogen (oospores) in presence of the fungicide have several advantages, including a precise and quantitative evaluation of the resistance status (Massi et al., 2021; Toffolatti & Vercesi, 2011). By scoring the number of germinated oospores and comparing to the untreated control, it is possible to quantify the percentage of resistant individuals at a discriminatory fungicide concentration (Toffolatti et al., 2007, 2018). For fungicides such as zoxamide, for which field resistance has not yet been reported, obtaining quantitative results can be very important because qualitative data tend to overestimate the resistance status (Collina, 2017).

This study reports the results obtained over three years of fungicide resistance monitoring activities on *P. viticola* oospores. A total number of 50 *P. viticola* populations from north-western Italy were characterized for their sensitivity to zoxamide and the results obtained during the experimental activities are reported below.

2. Materials and methods

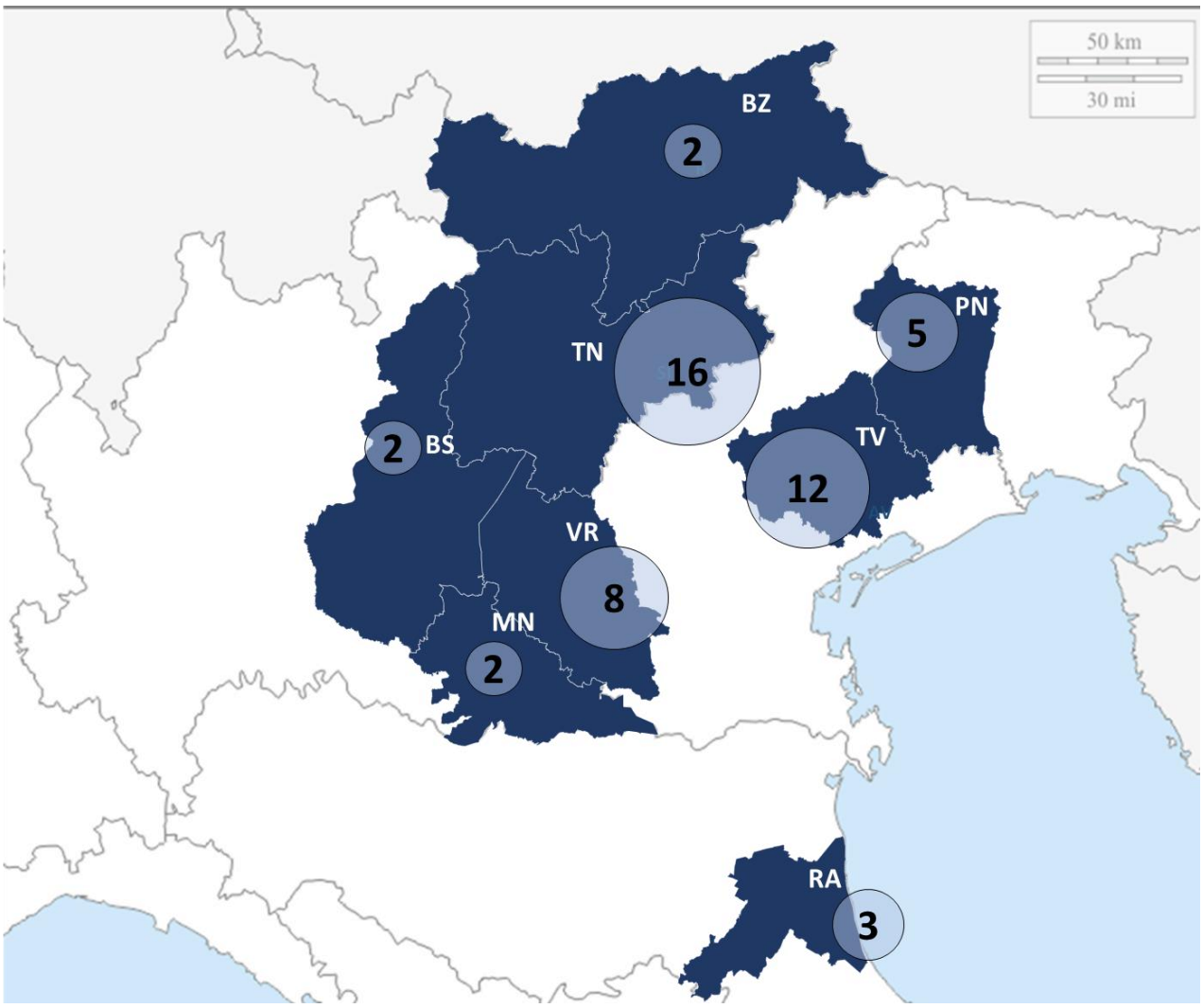
2.1 Sampling of *P. viticola* populations

The experimental vineyards sampled for oospores germination assays were selected in different regions of north-western Italy: Emilia-Romagna, Friuli, Lombardy, Trentino-Alto Adige and Veneto (Table 1). Grapevine leaves showing downy mildew mosaic symptoms were randomly collected in October 2018, 2019 and 2020 from fifty commercial vineyards located in heterogeneous geographic locations of the selected Italian regions (Figure 1). At least 100 grapevine leaves showing symptoms of downy mildew were collected from each vineyard depending on the disease incidence, and information on the treatments carried out in the corresponding growing seasons were collected in order to have an idea of the possible characteristics of the pathogen population. The number of treatments with zoxamide (always used in mixture with an anti-resistance partner) are reported in Table 1. Fungicide treatments were carried out using the farmers' equipment with commercial formulations at the doses indicated on the product labels.

Table 1: List of the 50 *P. viticola* populations sampled between 2018 and 2020.

Sample name	Region	Province code	Number of zoxamide applications in the corresponding sampling season	Sampling period
Z01	Veneto	VR	4	October 2018
Z02	Lombardy	BS	5	October 2018
Z03	Lombardy	MN	4	October 2018
Z04	Veneto	VR	5	October 2018
Z05	Veneto	VR	4	October 2018
Z06	Trentino-Alto Adige	TN	4	October 2018
Z07	Trentino-Alto Adige	TN	3	October 2018
Z08	Trentino-Alto Adige	TN	3	October 2018
Z09	Trentino-Alto Adige	TN	3	October 2018
Z10	Trentino-Alto Adige	BZ	3	October 2018
Z11	Trentino-Alto Adige	TN	2	October 2018
Z12	Friuli	TV	4	October 2018
Z13	Friuli	TV	3	October 2018
Z14	Friuli	PN	4	October 2018
Z15	Friuli	TV	5	October 2018
Z16	Friuli	PN	5	October 2018
Z17	Veneto	VR	4	October 2019
Z18	Lombardy	BS	5	October 2019
Z19	Lombardy	MN	4	October 2019
Z20	Veneto	VR	4	October 2019
Z21	Veneto	VR	3	October 2019
Z22	Trentino-Alto Adige	TN	4	October 2019
Z23	Trentino-Alto Adige	TN	2	October 2019
Z24	Trentino-Alto Adige	TN	3	October 2019
Z25	Trentino-Alto Adige	TN	3	October 2019
Z26	Trentino-Alto Adige	BZ	3	October 2019
Z27	Trentino-Alto Adige	TN	2	October 2019
Z28	Trentino-Alto Adige	TN	5	October 2019
Z29	Friuli	TV	4	October 2019
Z30	Friuli	TV	3	October 2019
Z31	Friuli	TV	3	October 2019
Z32	Friuli	TV	4	October 2019
Z33	Friuli	PN	4	October 2019
Z34	Veneto	VR	4	October 2020
Z35	Veneto	VR	3	October 2020
Z36	Trentino-Alto Adige	TN	3	October 2020
Z37	Trentino-Alto Adige	TN	3	October 2020
Z38	Trentino-Alto Adige	TN	3	October 2020
Z39	Trentino-Alto Adige	TN	3	October 2020
Z40	Friuli	TV	4	October 2020
Z41	Friuli	TV	4	October 2020
Z42	Friuli	TV	4	October 2020
Z43	Friuli	TV	4	October 2020
Z44	Friuli	TV	4	October 2020
Z45	Friuli	PN	5	October 2020
Z46	Friuli	PN	5	October 2020
Z47	Emilia-Romagna	RA	0	October 2020
Z48	Emilia-Romagna	RA	0	October 2020
Z49	Emilia-Romagna	RA	3	October 2020
Z50	Trentino-Alto Adige	TN	3	October 2020

Figure 1: Geographical distribution of Italian *P. viticola* populations sampled in north-western Italy. Numbers and size of the circles indicates the total number of populations sampled for each province, indicated on the map with alphabetic codes: Brescia (BS); Bolzano (BZ); Mantova (MN); Pordenone (PN), Ravenna (RA); Trento (TN); Treviso (TV); Verona (VR)



2.2 Sample processing and oospores sensitivity test

In laboratory, 50 fragments rich in oospores were cut from the leaves under microscope (Zeiss Primo Vert; Carl Zeiss, Milan, Italy), placed in nylon bags and stored for four months at 5 °C in the dark on a sandy substrate kept regularly watered (30 % water/sand weight), overwintering conditions that are highly favorable for the pathogen (Maddalena et al., 2021).

Oospore germinability was assessed on 1% water agar (Agar Noble, DIFCO) amended with different amounts of zoxamide (0, 0.01, 0.1, 0.2, 1, 2, 10, 20, 100, 200 and 400 mg/L) at 20 °C (Fig. 2). Zoxamide, technical grade, was dissolved in DMSO at 1 g/L concentration, diluted in double-distilled sterile water and added to sterile 1% water agar at 55 °C. DMSO concentration in the final medium was lower than 0.1 %, to avoid undesired effects on the oospore germination.

To perform germination assays, the oospores were isolated from the leaf debris , resuspended in sterile distilled water, counted, plated on the substrates and incubated in the dark at 20 °C (Toffolatti et al., 2007, 2018; Vercesi et al., 2010). Three plates containing four droplets of 100

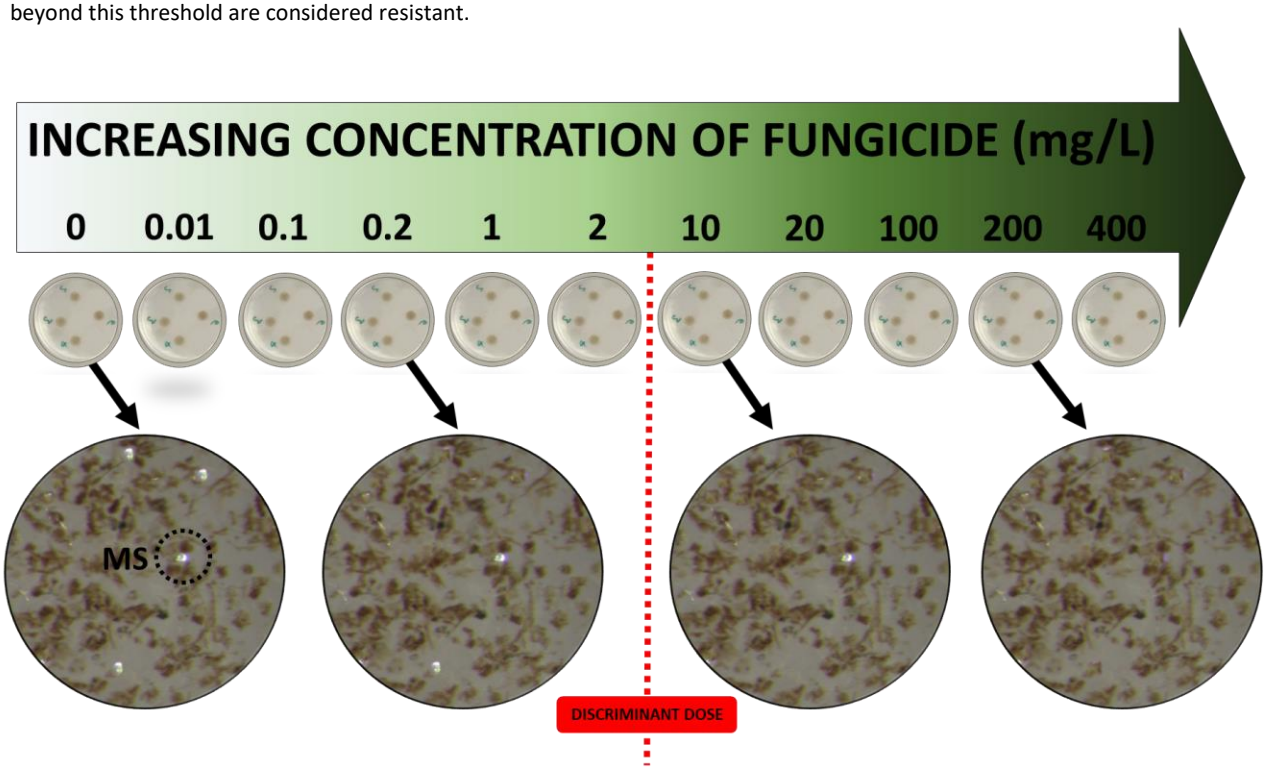
oospores each (replicates) were plated for a total number of 1200 oospores per sample. The number of macrosporangia formed at 7, 10 and 14 days after inoculation was counted to calculate the germination percentages (G), *i.e.* the percentage of germinated oospores over the total. The average values of G were used to calculate: i) the percentage of resistant oospores (RO), obtained by dividing the number of oospores germinating at the discriminatory concentration of 10 mg/L and oospores germinated at 0 mg/L ii) the germination inhibition percentage (GI) at each fungicide concentrations, calculated as:

$$GI = 100 - (\frac{G_x}{G_0} \times 100)$$

Where G_x is the mean germination percentage at the considered zoxamide concentration (x) and G_0 is the mean germination percentage on the untreated control medium.

The EC_{50}, *i.e.* the median fungicide concentration inhibiting the oospore germination, was calculated by probit analysis of GI values on log-transformed values of fungicide concentration (SPSS software version 24; IBM Analytics Italia, Milano, Italy) as reported by Toffolatti and coworkers (Toffolatti et al., 2018). The EC_{50} values obtained were used to calculate the resistance factor (RF), intended as the ratio between the EC_{50} value calculated for the tested population and the average EC_{50} value of the sensitive populations (Mair et al., 2016; Weber & Hahn, 2011). RFs > 10 were considered indicative of resistance.

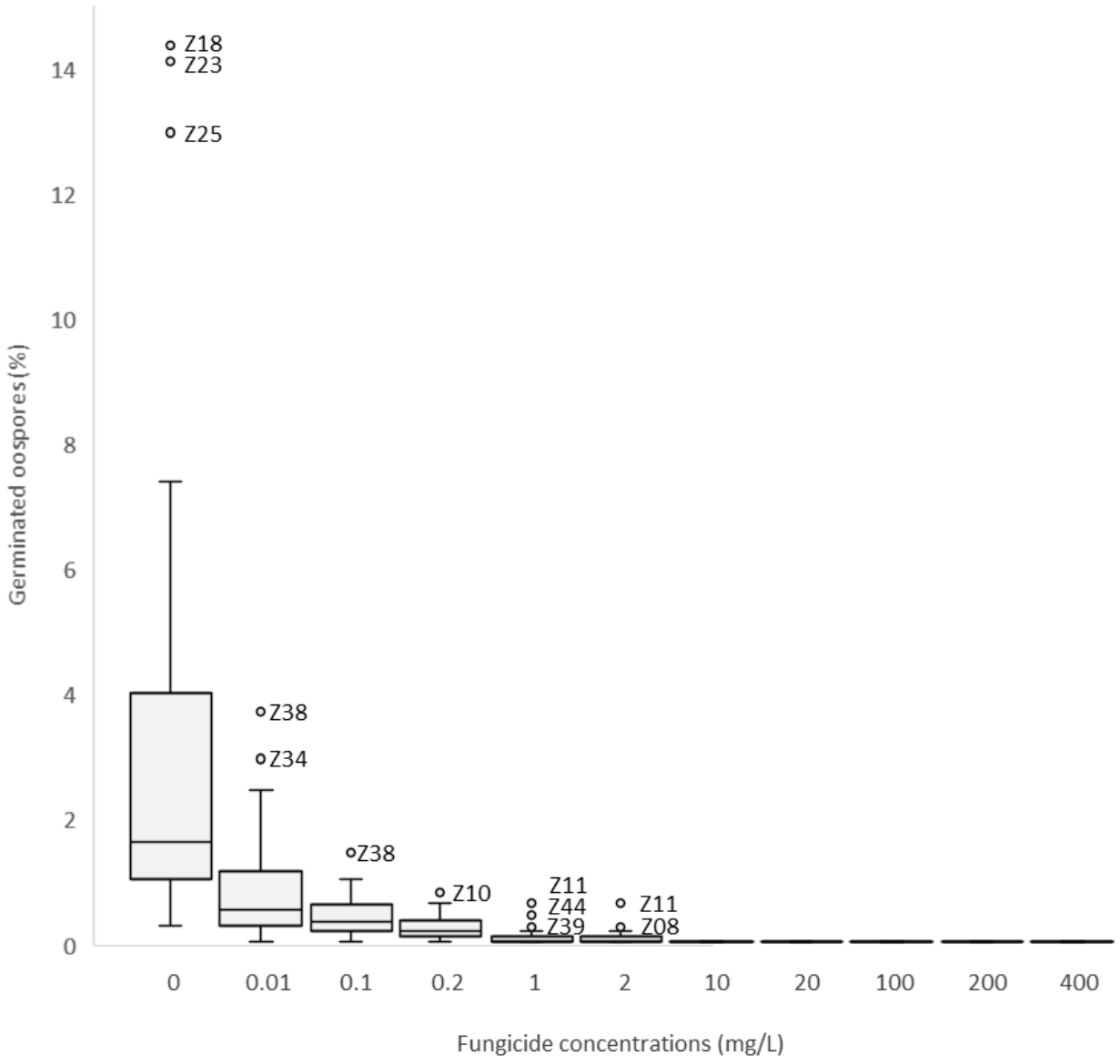
Figure 2: oospores plated on water agar amended with different amounts of zoxamide. The zoxamide concentrations adopted (mg / L) are shown inside the arrow. In the pictures below it is possible to see the plates containing the oospore droplets and the macrosporangia (MS) emerging from the oospores at 0, 0.2, 10 and 200 mg/L zoxamide. The red dashed line indicates the discriminating concentration of 10 mg/L used for the calculation of the percentage of resistant oospores: oospores germinated beyond this threshold are considered resistant.



3. Results

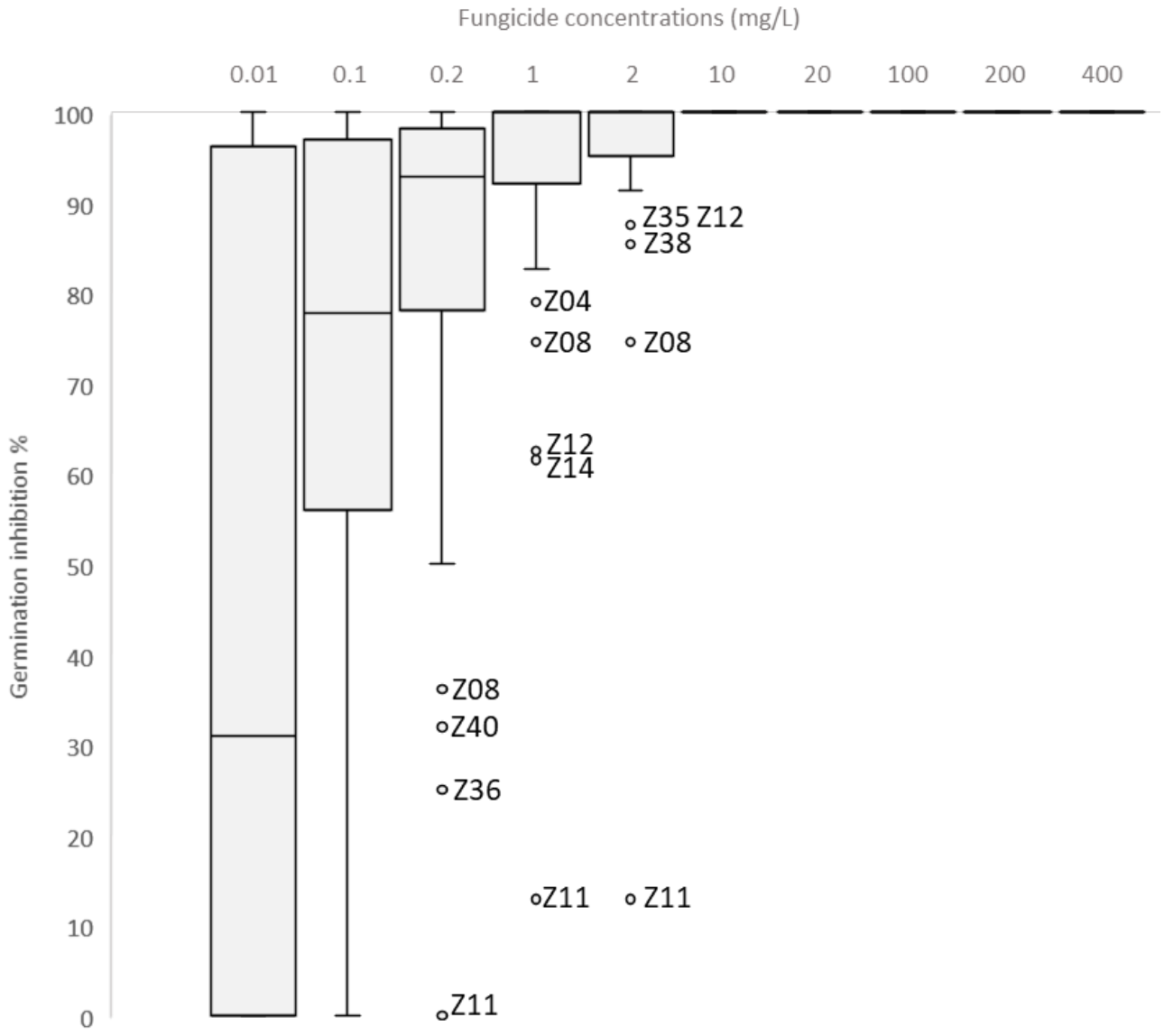
The germination percentages (G) of the oospores on the untreated controls ranged from a minimum of 0.25 to a maximum of 14.3%, with an average value of 3% (Figure 3). Of the 50 *P. viticola* population tested, three (Z18, Z23, Z25) showed percentages of germination over 12% in absence of the fungicide and were classified as outliers. All these populations were sampled in the second year of experimental activity (October 2019). A progressive decrease of G was observed at increasing concentrations of zoxamide in all the vineyards (Figure 3). Also in this case, some samples showed particularly high G rates and were classified as outliers. In particular sample Z11, collected in October 2018, showed very high G values also in presence of zoxamide (1 and 2 mg/L), and was classified as an outlier in the 2 out of 10 concentrations tested.

Figure 3: Box-plot distribution of the percentage of germinated oospores (G) at each zoxamide concentrations (mg/L) recorded during the experimental activity. Circles indicate outliers, codes indicate samples.



Increasing values of GI were recorded at increasing fungicide concentrations (Figure 4). GI increases from an average value of 45% at 0.01 mg/L to 100% at 10 mg/L for every sample tested. No germinated oospores were detected at the highest concentrations of 20, 100, 200 and 400 mg/L, allowing GI to reach the value of 100% for every sample tested. However, 9 populations (Z04, Z08, Z11, Z12, Z14, Z35, Z36, Z38 and Z40) showed a reduced GI at least in one of the tested zoxamide concentrations and were classified also in this case as outliers. In particular, five of them (Z08, Z11, Z12, Z35 and Z38) had GI values ranging from 13 to 85% at the zoxamide concentrations of 2 mg/L.

Figure 4. Box-plot distribution of the germination inhibition percentages (GI) at increasing zoxamide concentrations (mg/L) recorded during the experimental activity. Circles indicate outliers, codes indicate samples.



The EC_{50} values calculated from GI values were lower than 0.2 mg/L of zoxamide for all the samples tested (Table 2), with an average value of 0.022 mg/L. On the other hand, for nine (Z17, Z24, Z28, Z33, Z36, Z40, Z45, Z48 and Z50) of the 50 populations tested, it was not possible to calculate the EC_{50} values and RFs as no germinated oospores were detected beyond the first

fungicide concentration tested (0.01 mg/L). Consequently, it was not possible to fit the probit model for EC_{50} calculation on the collected data relating to these samples and the EC_{50} value was set at values lower than 0.01 mg/L.

Table 2: Sensitivity to zoxamide of the samples tested. EC_{50} with 95% confidence limits are reported in parentheses

SAMPLE	EC_{50} [mg/L]	RF
Z01	2.8×10^{-2} (2×10^{-2}; 3.9×10^{-2})	1.3
Z02	9×10^{-3} (5×10^{-3}; 1.3×10^{-2})	0.1
Z03	8×10^{-3} (0.1×10^{-5}; 4.7×10^{-2})	0.1
Z04	9.2×10^{-2} (3×10^{-2}; 2.4×10^{-1})	1.5
Z05	9.7×10^{-2} (7×10^{-3}; 2,5 x 10^{0})	1.6
Z06	2.3×10^{-2} (6×10^{-3}; 5.2×10^{-2})	0.4
Z07	1.3×10^{-3} (6.9×10^{-5}; 2.4×10^{-2})	0.0
Z08	1.5×10^{-1} (4.7×10^{-2}; 4.4×10^{-1})	2.5
Z09	1.5×10^{-2} (1.2×10^{-2}; 1.9×10^{-2})	0.2
Z10	2.2×10^{-4} (6.9×10^{-6}; 7.2×10^{-3})	0.0
Z11	8.4×10^{-3} (8×10^{-4}; 8.8×10^{-2})	0.1
Z12	3.2×10^{-2} (2.5x 10^{-2}; 4×10^{-2})	0.5
Z13	4×10^{-3} (1×10^{-3}; 6×10^{-3})	0.1
Z14	9.2×10^{-9} (1.5×10^{-12}; 5.4×10^{-5})	0.0
Z15	1.5×10^{-1} (1.2×10^{-1}; 2.3×10^{-1})	2.4
Z16	< 0.01	< 0.1
Z17	3.1×10^{-3} (9.63×10^{-4}; 1×10^{-2})	0.1
Z18	8.7×10^{-3} (3.7×10^{-3}; 2×10^{-2})	0.1
Z19	4.6×10^{-3} (1.6×10^{-3}; 1.3×10^{-2})	0.1
Z20	2.9×10^{-2} (1.6×10^{-2}; 5×10^{-2})	0.5
Z21	3.5×10^{-3} (1.1×10^{-3}; 1.1×10^{-2})	0.1
Z22	8.9×10^{-3} (3.7×10^{-3}; 2.1×10^{-2})	0.1
Z23	< 0.01	< 0.1
Z24	3.7×10^{-3} (1.2×10^{-3}; 1.1×10^{-2})	0.1
Z25	4.2×10^{-3} (1.4×10^{-3}; 1.2×10^{-2})	0.1

SAMPLE	EC_{50} (mg/L)	RF
Z34	4.5×10^{-3} (1.5×10^{-3}; 1.3×10^{-2})	0.1
Z35	< 0.01	< 0.1
Z36	3.1×10^{-2} (1.8×10^{-2}; 5.4×10^{-2})	0.5
Z37	1×10^{-3} (1.9×10^{-4}; 5.5×10^{-3})	0.0
Z39	3.9×10^{-2} (2.4×10^{-2}; 26.3×10^{-2})	0.6
Z41	2.2×10^{-2} (1.2×10^{-2}; 4.1×10^{-2})	0.4
Z42	< 0.01	< 0.1
Z44	1.1×10^{-4} (5.7×10^{-6}; 2.3×10^{-3})	0.0
Z45	8.8×10^{-3} (1.7×10^{-3}; 4.4×10^{-2})	0.1
Z47	< 0.01	< 0.1
Z48	1.8×10^{-3} (2.8×10^{-4}; 1.2×10^{-2})	0.0
Z49	1.9×10^{-3} (2.5×10^{-4}; 1,4 x 10^{-2})	0.0
Z50	2.4×10^{-4} (1.3×10^{-5}; 4.1×10^{-3})	0.0
Z52	< 0.01	< 0.1
Z53	9.8×10^{-4} (1.4×10^{-4}; 6.7×10^{-3})	0.0
Z54	5.9×10^{-3} (1.9×10^{-3}; 1.8×10^{-2})	0.1
Z56	2.6×10^{-3} (3.8×10^{-4}; 1.7×10^{-2})	0.0
Z57	3.1×10^{-2} (6.1×10^{-3}: 1.6×10^{-1})	0.5
Z58	< 0.01	< 0.1
Z59	2.1×10^{-4} (1.3×10^{-5}; 3.4×10^{-3})	0.0
Z60	5.13×10^{-2} (1.5×10^{-2}; 1.7×10^{-1})	0.8
Z61	< 0.01	< 0.1
Z62	1.5×10^{-3} (1.8×10^{-4}; 1.3x 10^{-2})	0.0
Z63	< 0.01	< 0.1

4. Discussion and conclusions

The results obtained from experimental activities carried out on samples collected in season 2018, 2019 and 2020 allowed to conclude that all the *P. viticola* populations monitored characterized by full sensitivity to zoxamide, as indicated by the very low EC_{50} values calculated. This assumption is also supported by the calculation of the resistance factors, all of which are widely below the indicative resistance threshold of 10 as proposed by Mair et al. and Weber and Hahn. However, the limited number of baseline sensitivity data to zoxamide for oospores germination test with which to make a comparison, makes the calculation and the interpretation of this value difficult. Moreover, it must be considered that many oospores in field retain the ability to germinate throughout more than one growing season (Kennelly et al., 2007), and the test method here adopted only provides short-term information on oospores germination, while the average life of these structures in the field is much longer. On the other hand, its use makes it possible to obtain data on the potential frequency of resistance in the primary inoculum for the following season and to evaluate the resistance status after the sexual reproduction stage of the pathogen (Vercesi et al., 2010).

The information here obtained permit a quantitative evaluation of the sensitivity status of the Italian *P. viticola* populations, proving useful data for their resistance monitoring and future monitoring activitiest. One of the cornerstone of interpreting fungicide resistance monitoring data is to make a comparisons with other data, obtained possibly with the same experimental method used in the considered study (Russel, 2004). In 2016, Toffolatti et al. performed a wide monitoring of resistance upon single-site fungicide, commonly utilized to control oomycetes, in Lombardy and Northern Italy (Toffolatti et al., 2016). This study represents an important point of reference, as part of the investigated panel of substances, zoxamide was evaluated too, and those experiments were conducted on oospores using the same methodology adopted in our work. Toffolatti and coworkers found that zoxamide successfully inhibited the germination of every collected sample of oospores at the concentrations tested, with EC_{50} values ranging from 0.0001 to 0.004 mg/L, even if in one sample the 0.08% of oospores germinated properly in presence of a zoxamide concentration of 10 mg/L. Although the average EC_{50} value of 0.022 mg/L obtained in our study is approximately 10 times higher than the maximum obtained by Toffolatti et al. in 2016, it must be noted the shifting is minimal compared to the well far field dose of 180 mg/L, and the detected sensitivity levels are very similar. Considering the further analogy of the monitored area, this suggests a resistance status of zoxamide which seems substantially stable in Norther Italy after 7 years. The slight differences here found can be reconducted to the different scale of monitoring, which in our case is more than three times larger, and to the different weather conditions of the years of sampling (ARPAL, 2021). All these factors can presumably affect in a minimal part the pathogen's response in terms of oospores germination. It is known indeed, that the amount of rainfall and the average temperatures occurring in vineyard up to 40–60 days before sampling, influence oospores germinability in laboratory conditions (Vercesi et al., 1999). Even within our data, we found a lower germination rates of the untreated controls of few samples and a particularly high rates in large part of the populations sampled in the second year, suggesting that there are dynamic fluctuations in oospore germination. This hypothesis is supported by other multi-year fungicide resistance studies on oospores, in which fluctuations in germination rates have been recorded from season to season (Toffolatti et al., 2018). However, the heterogeneous G values on the controls here detected did not influenced the result of the sensitivity tests, since

they were in line with the general G standard values (Maddalena et al., 2021; Toffolatti et al., 2007, 2015) and the dose response curve was adequate for EC_{50} calculation.

Considering the result obtained in this study it can be assumed that as far as it is known Italian *P. viticola* populations showed a good sensitivity to zoxamide, and it could be supposed that to date, in Italy, there are no reasonable evidences of *P. viticola* resistance shifting. Nonetheless, since this pathogen is particularly prone to mutation and adaptions in general (Gessler et al., 2011), additional care should be placed when operating with in-field populations. As for any other single-site fungicide, solo applications have to be reduced or better even avoided if possible; any improper application, such as an over-usage above the maximum number of treatments per season or reduced dose sprayings must be prevented (Bosch et al., 2014).

5. Bibliography

1. ARPAL. (2021). *Archivio metereologico Lombardia*. https://www.arpalombardia.it/Pages/Meteorologia/Osservazioni-e-Dati/Il-tempo-di-ieri/Archivio-Meteo-Giorno.aspx?firstlevel=Guida temperature minime e massime
2. Brent, K. J. (2012). Historical perspectives of fungicide resistance. In S. Tarlochan (Ed.), *Fungicide resistance in crop protection: Risk and management* (pp. 3–18). CABI.
3. Brent, K. J., & Hollomon, D. W. (2007). Fungicide resistance in crop pathogens. How can it be managed. In *FRAC Monograph No. 1* (2nd ed.). CropLife International.
4. Cai, M., Miao, J., Song, X., Lin, D., Bi, Y., Chen, L., Liu, X., & Tyler, B. M. (2016). C239S Mutation in the β-Tubulin of *Phytophthora sojae* Confers Resistance to Zoxamide. *Frontiers in Microbiology*, *7*, 762. https://doi.org/10.3389/fmicb.2016.00762
5. Calo, S., Billmyre, R. B., & Heitman, J. (2013). Generators of phenotypic diversity in the evolution of pathogenic microorganisms. *PLoS Pathogens*, *9*(3), e1003181. https://doi.org/10.1371/journal.ppat.1003181
6. Collina, M. (2017). Resistenza agli antiperonosporici, concetti chiave e prevenzione. No Title. *Supplemento A L'Informatore Agrario*, *13*, 8–10.
7. Corio-Costet, M.-F. (2015). Monitoring resistance in obligate pathogens by bioassays relating to field use: grapevine powdery and downy mildews. In *Fungicide Resistance in Plant Pathogens*. Springer Japan. https://doi.org/10.1007/978-4-431-55642-8_16
8. FRAC. (2020). *Resistance overview*. www.frac.info
9. FRAC. (2021). *FRAC Code List ©*2021*. https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2021--final.pdf?sfvrsn=f7ec499a_2
10. Gessler, C., Pertot, I., & Perazzolli, M. (2011). *Plasmopara viticola*: A review of knowledge on downy mildew of grapevine and effective disease management. *Phytopathologia Mediterranea*, *50*(1), 3–44. https://doi.org/10.14601/Phytopathol_Mediterr-9360
11. Kennelly, M. M., Gadoury, D. M., Wilcox, W. F., Magarey, P. A., & Seem, R. C. (2007). Primary infection, lesion productivity, and survival of sporangia in the grapevine downy mildew pathogen *Plasmopara viticola*. *Phytopathology*, *97*(4), 512–522. https://doi.org/10.1094/PHYTO-97-4-0512
12. Maddalena, G., Russo, G., & Toffolatti, S. L. (2021). The study of the germination dynamics of *Plasmopara viticola* oospores highlights the presence of phenotypic synchrony with the host. *Frontiers in Microbiology*, *12*, 698586. https://doi.org/10.3389/fmicb.2021.698586
13. Mair, W. J., Deng, W., Mullins, J. G. L., West, S., Wang, P., Besharat, N., Ellwood, S. R., Oliver, R. P., & Lopez-Ruiz, F. J. (2016). Demethylase inhibitor fungicide resistance in *Pyrenophora teres* f. sp. *teres* associated with target site modification and inducible overexpression of *Cyp51*. *Frontiers in Microbiology*, *7*, 1279. https://doi.org/10.3389/fmicb.2016.01279
14. Massi, F., Torriani, S. F. F., Borghi, L., & Toffolatti, S. L. (2021). Fungicide resistance evolution and detection in plant pathogens: *Plasmopara viticola* as a case study.

Microorganisms, *9*(1), 1–18. https://doi.org/10.3390/microorganisms9010119
15. Ruggiero, P., & Regiroli, G. (2000). Zoxamide, a novel fungicide for vines and vegetables. *Atti, Giornate Fitopatologiche Volume 2*.
16. Russel, P. (2004). Sensitivity baselines in fungicide resistance research and management. In *FRAC Monograph 3*. CropLife International.
17. Toffolatti, S. L., & Vercesi, A. (2011). QoI resistance in *Plasmopara viticola* in Italy: Evolution and management strategies. In *Fungicide Resistance in Crop Protection: Risk and Management* (pp. 172–183). https://doi.org/10.1079/9781845939052.0172
18. Toffolatti, S. L, Serrati, L., Sierotzki, H., Gisi, U., & Vercesi, A. (2007). Assessment of QoI resistance in *Plasmopara viticola* oospores. *Pest Management Science*, *63*(2), 194–201. https://doi.org/https://doi.org/10.1002/ps.1327
19. Toffolatti, S.L, Venturini, G., Campia, P., Cirio, L., Bellotto, D., & Vercesi, A. (2015). Sensitivity to cymoxanil in Italian populations of *Plasmopara viticola* oospores. *Pest Management Science*, *71*(8), 1182—1188. https://doi.org/10.1002/ps.3906
20. Toffolatti, Silvia Laura, Campia, P., Venturini, G., & Vercesi, A. (2016). Monitoraggio della resistenza ai principali fungicidi antiperonosporici in Lombardia. *Giornate Fisiopatologiche*.
21. Toffolatti, Silvia Laura, Russo, G., Campia, P., Bianco, P. A., Borsa, P., Coatti, M., Torriani, S. F., & Sierotzki, H. (2018). A time-course investigation of resistance to the carboxylic acid amide mandipropamid in field populations of *Plasmopara viticola* treated with anti-resistance strategies. *Pest Management Science*, *74*(12), 2822—2834. https://doi.org/10.1002/ps.5072
22. Vercesi, A., Toffolatti, S. L., Zocchi, G., Guglielmann, R., & Ironi, L. (2010). A new approach to modelling the dynamics of oospore germination in *Plasmopara viticola*. *European Journal of Plant Pathology*, *128*(1), 113–126. https://doi.org/10.1007/s10658-010-9635-8
23. Vercesi, A., Tornaghi, R., Sant, S., Burrano, S., & Faoro, F. (1999). A cytological and ultrastructural study on the maturation and germination of oospores of *Plasmopara viticola* from overwintering vine leaves. *Mycological Research*, *103*(2), 193–202. https://doi.org/10.1017/S095375629800700X
24. Weber, R. W. S., & Hahn, M. (2011). A rapid and simple method for determining fungicide resistance in *Botrytis*. *Journal of Plant Diseases and Protection*, *118*(1), 17–25. http://www.jstor.org/stable/43229155
25. Young, D. H., & Slawecki, R. A. (2001). Mode of Action of Zoxamide (RH-7281), a new oomycete fungicide. *pesticide biochemistry and physiology*, *69*(2), 100–111. https://doi.org/https://doi.org/10.1006/pest.2000.2529
26. Ziogas, B. N., Markoglou, A. N., Theodosiou, D. I., Anagnostou, A., & Boutopoulou, S. (2006). A high multi-drug resistance to chemically unrelated oomycete fungicides in *Phytophthora infestans*. *European Journal of Plant Pathology*, *115*(3), 283–292. https://doi.org/10.1007/s10658-006-9007-6

Characterization of Italian *Plasmopara viticola* populations for resistance to oxathiapiprolin

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Abstract: Oxathiapiprolin is a novel fungicide and the first of the piperidinyl thiazole isoxazoline class to be discovered. This fungicide has been reported to have high activity against *Plasmopara viticola*, the grapevine downy mildew agent, and other plant-pathogenic oomycetes. In this study, the baseline sensitivity of Italian *P. viticola* populations towards oxathiapiprolin was established on 29 populations collected in ten different viticultural areas. The populations were characterize for their sensitivity profiles through biological and molecular assays. Oxathiapiprolin exhibited substantial inhibitory activity against 27 of the 29 populations tested, with EC_{50} values ranging from a minimum under 4 x 10^{-5} mg/L to over 4 x 10^{-1} mg/L, with an average value of 3.2 x 10^{-02} mg/L. Two stable suspected oxathiapiprolin-resistant mutants were isolated from population exhibiting reduced sensitivity and sequenced for the oxathiapiprolin target gene *PvORP1.* Comparison with wild-type isolates revealed that less sensitive isolates possessed a heterozygous mutation causing the amino acid substitution N837I previously reported in the literature. The results obtained suggest that the risk of Italian *P. viticola* populations to develop resistance to oxathiapiprolin is concrete, and that the efficacy of this molecule should be well monitored in future to preserve its effectiveness.

1. Introduction

Grapevine downy mildew, caused by the phytopathogenic oomycete *Plasmopara viticola* (Berk. *et* Curt.) Berl. & De Toni, is one of the major threats to grapevine production worldwide. Severe disease epidemics caused by this oomycete are often associated with consistent quantitative and qualitative yield losses (Gessler et al., 2011). *P. viticola* is a native species from North America and it causes the main damage to *Vitis vinifera* L. (the Eurasian grapevine species), which is the most cultivated grapevine species due to the high quality of its grapes. Considering the high susceptibility of *V. vinifera* cultivars towards this pathogen (Lafon & Clerjeau, 1988), the growing of traditional varieties is not conceivable without frequent fungicide applications, and chemical control of the pathogen still represents the most important measure to ensure an adequate yield (Toffolatti et al., 2018).

Repeated treatments with selectively active site-specific fungicides are often followed by an acquired and hereditary reduction in the sensitivity of the fungus to the specific antifungal agent. This phenomenon is known as fungicide resistance (Background Information, www.frac.info), and affects many single/oligo-site active ingredients actually available for chemical control of grapevine downy mildew (Massi et al., 2021). The main reason why many target fungicides are affected by fungicide resistance is that for these active substances, single nucleotide polymorphisms (SNPs) in the gene encoding the target proteine could cause a decrease in sensitivity (Ma & Michailides, 2005). In order to preserve the effectiveness of such compounds, fungicide resistance must be carefully managed, and to this purpose, the monitoring of *P. viticola* populations for their sensitivities to the different active substances plays a key role in resistance management (Brent & Hollomon, 2007).

Oxathiapiprolin (OXTP) was the first of the piperidinyl thiazole isoxazoline fungicides to be discovered (Pasteris et al., 2016), and has been shown to be highly effective against a large number of plant pathogenic oomycetes, including *P. viticola* (Cohen, & Csinos, 2015; Ji et al., 2014; Miao, Dong, et al., 2016). Binding assays and affinity chromatography carried out on OXTP have shown that the intracellular target of this fungicide is one of the members of the oxysterol binding protein (OSBP)-related proteins (*ORPs*) family (Andreassi et al., 2013; Miao et al., 2016; Pasteris et al., 2016). Although in oomycetes the precise function of *ORPs* is not clear, this family of proteins is involved in a wide range of functions in all eukaryotes, including intracellular lipid metabolism, sterol transport and signal transduction (Raychaudhuri & Prinz, 2010). One of the main factors on which the assessment of fungicide resistance risk is focused is the establishment of baseline data, which define the level of sensitivity of a population never exposed to the fungicide under investigation, as well as the selection of resistant individuals. The availability of these data allows a comparison with the data obtained from suspected resistant isolates and is essential in planning and implementing anti-resistance strategies to manage fungicide resistance (Russel, 2004).

To date there are few data available regarding the potential of *P. viticola* to develop resistance to OXTP , or any reports of possible single nucleotide polymorphisms (SNPs) in the gene encoding for the fungicide target linked to a possible decrease in sensitivity (Mboup et al., 2022; Miao, Dong, et al., 2016; Pasteris et al., 2016). In particular, Mboup et al. (2022) reported reduced sensitivity of *P. viticola* field isolates linked to three possible nucleotide polymorphisms changing the amino acid sequence at position L863, N837 or G770 in the OSBP gene.

The objectives of the current study were to: (I) establish a baseline sensitivity of *P. viticola* Italian field populations to OXTP and (II) investigate OXTP-resistance mechanism by sequencing and comparing the *ORP* gene (*PvORP1*) of wild-type and suspected resistant *P. viticola* isolates.

2. Materials and methods:

2.1 Sampling

Leaves showing downy mildew symptoms were collected in 2019 and 2020 from twenty-nine commercial vineyards located in ten different provinces of Italy (Figure 1). At least 50 grapevine leaves were collected from each vineyard at different times of the grapevine growing season (Table 1), depending on the availability of inoculum. Information on the number of treatments

carried out with OXTP during the season were collected in order to have an idea of the possible selection pressure exerted by the fungicide on the pathogen population (Table 1). Fungicide treatments were carried out using the farmers' equipment with commercial formulations at the doses indicated on the product labels. Of the 29 *P. viticola* populations, 12 were collected from vineyards treated 1-3 times with OXTP, two from vineyards where OXTP was applied 4 times and 15 from fields where OXTP has not been applied during the monitoring year. However, in one of these latter vineyards (Pv-26) OXTP was applied for five consecutive years prior to 2019.

Figure 1: Geographical distribution of Italian *P. viticola* populations sampled. Numbers and size of the circles indicates the total number of populations sampled for each Italian province, indicated on the map with alphabetic codes: Avellino (AV); Brindisi (BR); Firenze (FI); Pescara (PE) Roma (RM); Siena (SI); Pordenone (PN); Pavia (PV); Trento (TN); and Verona (VR).

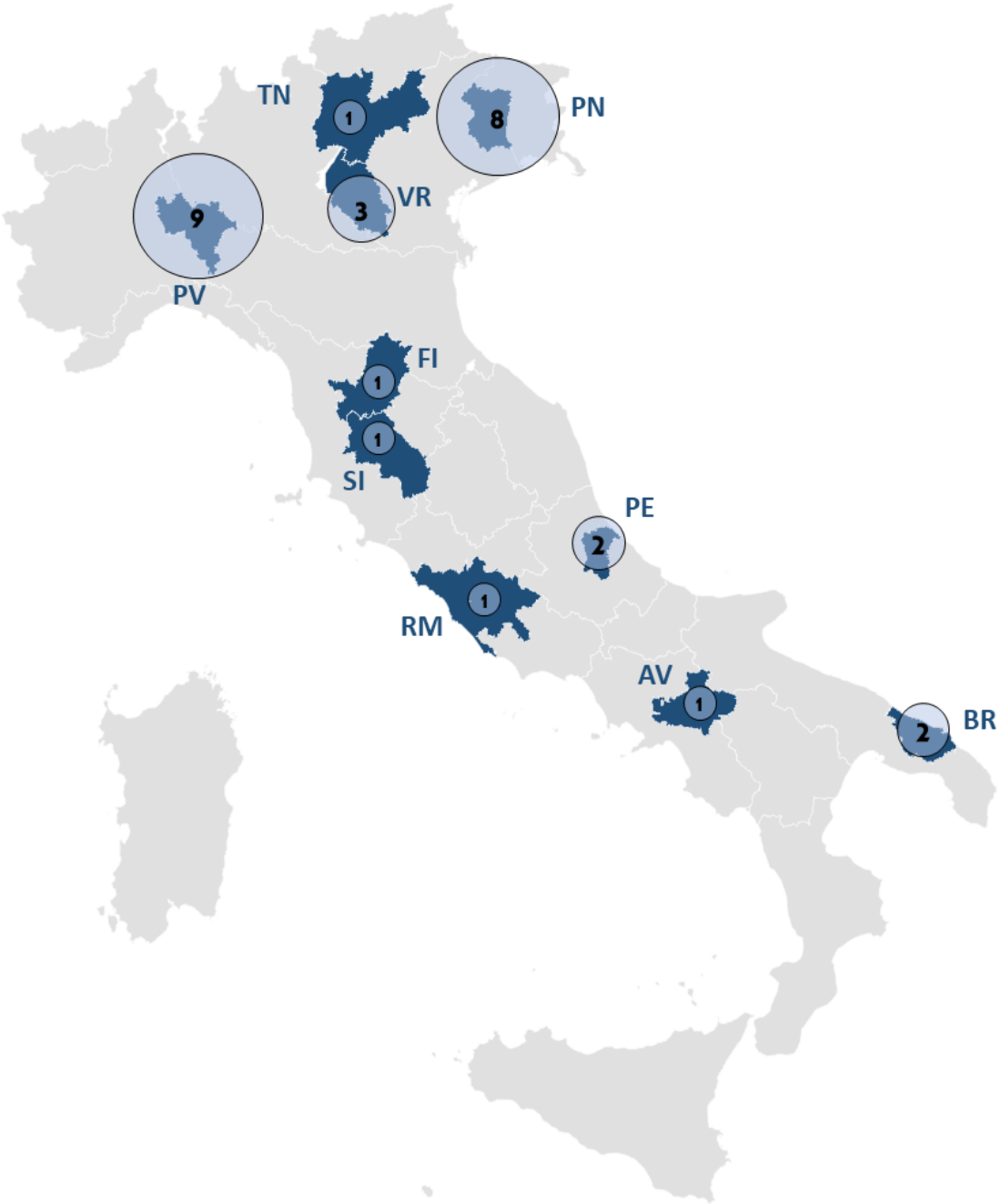


Table 1: Sample code, period and year of sampling, locations codes according to Figure 1 and total number of OXTP applications performed in mixture with fungicides belonging to different chemical classes during the sampling season.

Sample code	Sampling period* and year	Province code	Region	Number of treatments with OXTP
Pv-01	BS, 2019	PV	Lombardy	0
Pv-02	MS, 2019	PV	Lombardy	0
Pv-03	MS, 2019	PV	Lombardy	3
Pv-04	MS, 2019	PV	Lombardy	3
Pv-05	BS, 2019	PN	Friuli	0
Pv-06	MS, 2019	PN	Friuli	0
Pv-07	MS, 2019	PN	Friuli	2
Pv-08	MS, 2019	PN	Friuli	2
Pv-09	MS, 2019	PE	Abruzzo	4
Pv-10	MS, 2019	VR	Veneto	0
Pv-11	ES, 2019	AV	Campania	0
Pv-12	ES, 2019	RM	Lazio	2
Pv-13	ES, 2019	BR	Puglia	0
Pv-14	BS, 2020	PN	Friuli	0
Pv-15	ES, 2020	PN	Friuli	0
Pv-16	ES, 2020	PN	Friuli	2
Pv-17	ES, 2020	PN	Friuli	2
Pv-18	BS, 2020	PV	Lombardy	0
Pv-19	MS, 2020	PV	Lombardy	0
Pv-20	ES, 2020	PV	Lombardy	0
Pv-21	ES, 2020	PV	Lombardy	1
Pv-22	ES, 2020	PV	Lombardy	1
Pv-23	ES, 2020	PE	Abruzzo	4
Pv-24	ES, 2020	VR	Veneto	2
Pv-25	ES, 2020	VR	Veneto	2
Pv-26	ES, 2020	BR	Puglia	0
Pv-27	ES, 2020	TN	Trentino-Alto Adige	2
Pv-28	ES, 2020	SI	Toscana	0
Pv-29	ES, 2020	FI	Toscana	0

*BS (beginning of growing season, from May to June); *MS (mid growing season, from July to august) *ES (end of growing season, from September to October)

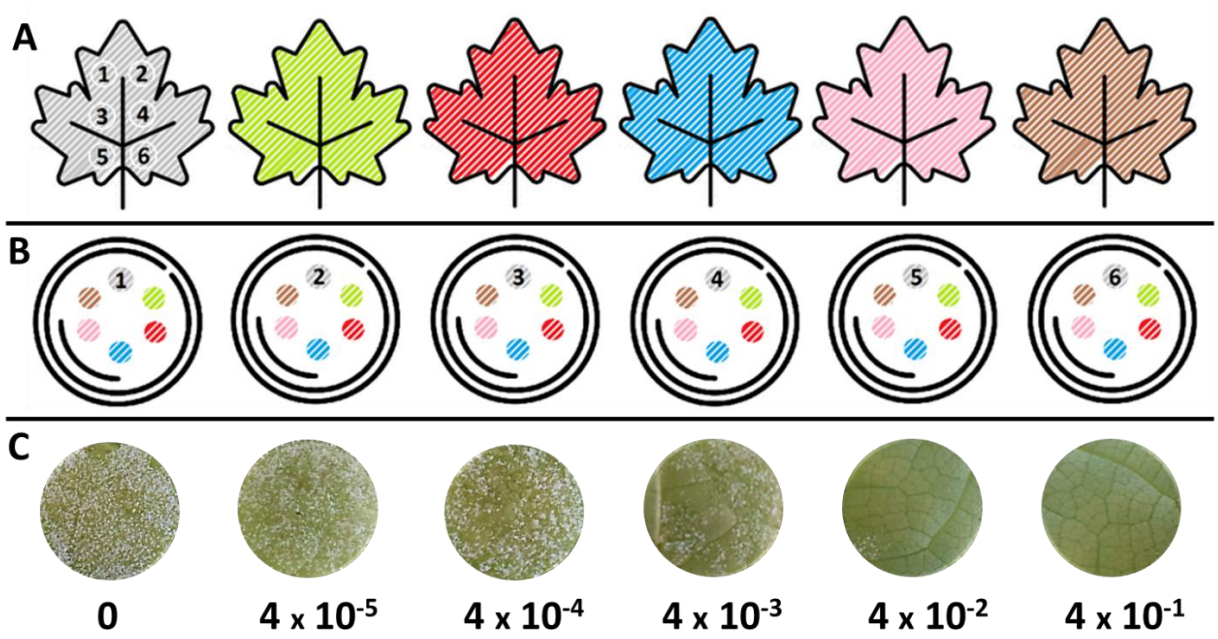
2.2 Sensitivity assays

The collected leaves were transferred to the laboratory in refrigerated bags, washed under running tap water, placed in a humid chamber and incubated overnight at 20–22 °C. Newly produced sporangia were resuspended in water: part of the suspension was immediately used for the sensitivity assay and the rest was kept at -20 °C until DNA extraction.

The fungicide sensitivity assays were carried out following the PLASVI OSBPI (*Plasmopara viticola*) microtiter plate test described by FRAC (FRAC, 2021), adjusting the sporangial suspension to $5x10^4$ sporangia mL^{-1}. In brief, six leaf discs (1.5 cm diameter) per fungicide concentration were placed, with the lower side upwards, in a Petri dish with moistened paper and sprayed with the fungicide prior to inoculation with *P. viticola* (Figure 2). A test set for one population (including untreated control) consists therefore of six Petri dishes, one for each of the six fungicide concentrations, containing six leaf discs. OXTP, technical grade (96.7%, active ingredient [a.i.]), was kindly provided by Syngenta Crop Protection AG Research Center (Stein, Switzerland). The fungicide was accurately weighed and dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, Milano, Italy) to

prepare a 1000 mg/L stock solution, which was stored in darkness at 4°C until serially dilution in double-distilled sterile water (ddH_2O) to obtain the desired fungicide concentrations for sensitivity test. The fungicide concentrations used for field isolates were 4 x 10^{-5}, 4 x 10^{-4}, 4 x 10^{-3}, 4 x 10^{-2}, 4 x 10^{-1} mg/L, and the final concentration of DMSO was always below 0.1% (v/v).

Figure 2: Schematic representation of the sensitivity test performed on the sporangia suspensions obtained from the populations under investigation: six leaf discs originating from six different cv. Pinot-noir leaves were cut out with a cork borer (A) and placed with the lower side upwards in six different Petri dishes containing moistened paper (B). The leaf discs were sprayed with increasing concentration of OXTP (concentrations reported below mg/L), led dry under the hood and then inoculated with *P. viticola.* To estimate the disease severity, each leaf disc was scored for the area affected by sporulation 9 days after inoculation (C).



After fungicide spraying, the treated leaf discs were dried in a flow hood and inoculated with the sporangia suspensions by equally spraying the suspension onto the leaf discs and incubated in a humid chamber at 20–22 °C with a 12:12 h photoperiod. Each leaf disc was scored for the area affected by sporulation 9 days after inoculation, and disease severity, expressed as an average percentage index of infection (I%I) (Toffolatti et al., 2018), was calculated for each fungicide concentration. Sporulation inhibition (IS) was expressed as a percentage calculated as

$$IS = 100 - \left(\frac{I\%I_x}{I\%I_0} \times 100\right)$$

where $I\%I_x$ is the I%I at a single OXTP concentration (x) and $I\%I_0$ is the I%I in the absence of the fungicide (untreated control).

The half maximal effective concentration (EC_{50}), i.e. the median fungicide concentration inhibiting sporulation of *P. viticola* on leaf discs, was calculated by probit analysis (Toffolatti et al., 2018) of IS values on log-transformed values of fungicide concentration (SPSS software version 24; IBM Analytics Italia, Milano, Italy). The EC_{50} values were used to calculate the resistance factor (RF), intended as the ratio between the EC_{50} value calculated for the tested population and the average

EC_{50} value of sensitive isolates (Mair et al., 2016; Weber & Hahn, 2011). RFs > 10 were considered indicative of resistance (Weber & Hahn, 2011).

2.3 Isolation of *P. viticola* strains resistant to oxathiapiprolin

Based on preliminary results achieved on the fungicide tests on *P. viticola* populations, the concentration of 0.4 mg/L OXTP was tentatively considered the discriminatory dose for the identification of resistant isolates. Of the 29 *P. viticola* populations tested, only three (Pv-16, Pv-24 and Pv-26) showed sporulation at this concentration and only from one of them (Pv-16), two stable single-sporangia strains (Pv-16.1 and Pv-16.2) were successfully isolated at this discriminatory concentration.

Single-sporangia strains were obtained by serially diluting a sporangia suspension prepared as described by Toffolatti and coworkers (Toffolatti et al., 2018). Briefly, the sporangia suspension was obtained by an individual sporangiophore and serially diluted on untreated leaves (cv Pinot noir) which were incubated as previously described. The individual sporangiophores were isolated under stereo microscope (TiEsseLab NSZ 800 FL) picking them up with a sterile pincer and depositing them in a 20 µL water droplet dispensed on the underside of a leaf. Serial dilution of sporangia was carried out by inoculating 5 µL of the suspension in 20 µL of water for five times. The presence of a single sporangium in the droplet was verified at the microscope. At the end of the incubation period, fresh sporangia were collected and propagate on fresh leaves to maintain the strain and collect sporangia for sensitivity assays and DNA extraction. The sensitivity profile of the isolates was assessed as previously described, by adding the concentration of 4 mg/L OXTP. The remaining sporangia suspension was stored at -20 °C until DNA extraction.

2.4 DNA extraction and *PvORP1* sequencing

DNA was extracted from suspected resistant strains and from 24 sensitive reference isolates never exposed to OXTP belonging to the collection of the Department of Agricultural and Environmental Sciences (Disaa, University of Milan), using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. DNA quality and concentration were spectrophotometrically determined (Nanodrop ND1000; Thermo Fisher Scientific, Rodano, Milan, Italy).

Primers for PCR amplifications of a portion (705 bps) of the gene coding for OSBP synthetized from Microsynth, (Balgach, Switzerland) are reported in Table 2. Amplification was performed in an Eppendorf Mastercycler Ep (Eppendorf, Milano, Italy) thermocycler a total volume of 50 µL containing 1x Dream Taq Green PCR Master Mix (Thermo-Fisher Scientific), 0.5 µM of the primers listed in Table 2, and 50 ng of DNA. Negative controls (water) were included. The following PCR conditions were used: first 5 min initial denaturation at 94 °C, then 34 cycles of 30 s at 94 °C, 30 s at 55.3 C°, 60 s at 72 C° and finally a 5 min extension step at 72°. Amplified DNA was purified and sequenced (Sanger sequencing) by Eurofins Genomics (Vimodrone, Milano, Italy) and Sequencher 5.4.6 software (Gene Codes Corporation, Ann Arbor, MI, USA) was used to compare the amino acid sequence of the *PvORP1* gene from wild-type reference isolates and the OXTP suspected resistant mutants.

Table 2: List of the primers used for genotyping resistance to OXTP, sequence of the primers, annealing temperatures (T_a), and direction.

Primer	Sequence (5'-3')	T_a (°C)	Strand
Pv1603	AAC GTT GCG TAT TCA CAA GA	55.3°	forward
Pv1606	ATC TGT GGG TGT CTT GGA		reverse

3. RESULTS

3.1 Sensitivity assays

Globally, the disease severity index on the untreated controls (I%I) ranged from 26.2 to 100 %, with an average value of 74.8 % (Table 3). However, in 28 of the 29 populations tested this value never dropped below 45%, and the only sample outside this range was PV-04, where I%I reached the maximum of only 26.2%.

In general, OXTP exhibited a progressive and strong inhibitory effect on *P. viticola* sporangia at increasing concentrations, as indicated by the high values of IS observed between 0.00004 and 0.4 mg/L of active substance (Table 3). At the lowest concentration (4 x 10^{-5} mg/L), more than 20% of the tested samples were already inhibited over 50%. This percentage reaches 58% and 93% at 4 x 10^{-3} and 4 x 10^{-2} mg/L of OXTP respectively. Only three samples (Pv-16, PV-24 and PV-26) were able to sporulate at the maximum concentration of 4 x 10^{-1} mg/L, and only one of them (PV-26) was not inhibited over 50%.

Table 3: Disease severity (I%I) on the untreated control, sporulation inhibition (IS) at each OXTP concentrations (0.00004-0.4 mg/L), EC_{50} values with 95% confidence limits in parentheses and resistance factors (RF) of *P. viticola* field populations analyzed during the experimental activities.

Sample code	I%I (%)	IS (%)					EC_{50} (mg/L)	RF
	0	0.00004	0.0004	0.004	0.04	0.4		
Pv-01	50	14.9	33.3	43.3	100	100	7.11 X 10^{-03} (9.99 X 10^{-04}; 5.07 X 10^{-02}) [a]	1.5
Pv-02	66.7	15.0	14.9	85.7	100	100	7.72 X 10^{-04} (2.69 X 10^{-04}; 2.22 X 10^{-03})	0.2
Pv-03	71.4	15.0	14.9	83.3	100	100	8.68 X 10^{-04} (2.94 X 10^{-04}; 2.56 X 10^{-03})	0.2
Pv-04	26.2	15.0	14.9	72.7	100	100	1.49 X 10^{-03} (4.42 X 10^{-04}; 5.01 X 10^{-03})	0.3
Pv-05	83.3	0.0	82.9	94.3	100	100	< 0.00004	< 0.1
Pv-06	78.6	33.3	69.7	84.8	100	100	1.24 X 10^{-04} (2.93 X 10^{-05}; 5.27 X 10^{-04})	< 0.1
Pv-07	85.7	13.9	38.9	50.0	72.2	100	2.92 X 10^{-03} (5.53 X 10^{-04}; 1.54 X 10^{-02})	0.6
Pv-08	95.2	12.5	27.5	45.0	90.0	100	2.02 X 10^{-03} (5.75 X 10^{-04}; 7.08 X 10^{-03})	0.4
Pv-09	83.3	25.7	28.6	92.1	100	100	3.38 X 10^{-04} (1.13 X 10^{-04}; 1.01 X 10^{-03})	0.1
Pv-10	100	7.1	23.8	23.8	71.4	100	1.10 X 10^{-02} (2.45 X 10^{-03}; 4.92 X 10^{-02})	2.3
Pv-11	90.5	26.3	36.8	39.5	68.4	100	4.57 X 10^{-03} (3.96 X 10^{-04}; 5.27 X 10^{-02})	1.0
Pv-12	57.1	75.0	33.3	75.0	91.7	100	< 0.00004	< 0.1
Pv-13	90.5	31.6	28.9	34.2	92.1	100	1.40 X 10^{-03} (2.96 X 10^{-04}; 6.64 X 10^{-03})	0.3
Pv-14	90.5	76.3	76.3	78.9	100	100	< 0.00004	< 0.1
Pv-15	90.5	42.1	81.6	76.3	76.3	100	< 0.00004	< 0.1
Pv-16	76.2	43.8	9.4	59.4	59.4	71.9	4.80 X 10^{-03} (3.12 X 10^{-04}; 7.38 X 10^{-02})	1.0
Pv-17	92.9	30.8	23.1	38.5	69.2	100	6.96 X 10^{-03} (6.21 X 10^{-04}; 7.80 X 10^{-02})	1.5
Pv-18	64.3	37.0	51.9	74.1	77.8	100	2.41 X 10^{-04} (2.54 X 10^{-05}; 2.28 X 10^{-03})	0.1

Pv-19	71.4	63.3	46.7	43.3	93.3	100	8.62×10^{-05} (6.52×10^{-06}; 1.14×10^{-03})	< 0.1
Pv-20	59.5	68.6	68.6	73.5	100	100	< 0.00004	< 0.1
Pv-21	57.1	15.8	21.1	36.8	52.6	100	3.28×10^{-02} (3.21×10^{-03}; 3.34×10^{-01})	7.0
Pv-22	78.6	93.3	93.3	94.9	96.1	100	< 0.00004	< 0.1
Pv-23	50	4.8	19.0	9.5	85.7	100	8.93×10^{-03} (2.54×10^{-03}; 3.14×10^{-02})	1.9
Pv-24	88.1	13.5	10.8	13.5	40.5	54.1	4.01×10^{-01} (3.31×10^{-02}; $5.19 \times 10^{+00}$)	85.7
Pv-25	73.8	32.3	19.4	58.1	58.1	100	6.57×10^{-03} (4.39×10^{-04}; 9.82×10^{-02})	1.4
Pv-26	78.6	3.0	6.1	3.0	30.3	33.3	> 0.4	> 85.7
Pv-27	95.2	72.5	72.5	65.0	82.5	100	< 0.00004	< 0.1
Pv-28	81.0	38.2	50.0	52.6	100	100	1.07×10^{-03} (1.26×10^{-05}; 9.04×10^{-02})	0.2
Pv-29	45.2	15.8	21.1	36.8	52.6	100	3.28×10^{-02} (3.21×10^{-03}; 3.34×10^{-01})	7.0

The EC_{50} profiles calculated from IS values ranged from a minimum under 4×10^{-5} mg/L to a maximum over 4×10^{-1} mg/L, with an average value of 3.2×10^{-02} mg/L (Table 3; Figure 2). In most of the samples tested, the values calculated were very low, indicating a typical situation of sensitivity. In particular, for 7 samples (Pv-05, Pv-12, PV-14, PV-15 PV-22 and Pv-27) the EC_{50} values obtained were below the lowest OXTP concentration tested (0.00004 mg/L). On the other hand, PV-24 and PV-26 showed particularly high values. For the former it was estimated an EC_{50} value which coincides with the maximum concentration of OXTP (4×10^{-1} mg/L), whereas for the latter the EC_{50} values obtained was higher than the concentration range tested.

The general situation of sensitivity well described from the values mentioned above, is confirmed by almost all the resistance factors calculated, which are extremely low and for 16 samples even below 0.1 (Table 3). The only samples with RFs>10 were Pv-24 and Pv-26.

The two single strains Pv-16.1 and Pv-16.2, isolated from survivors of sensitivity test performed on suspected resistant population Pv-16, exhibited reduced sensitivity to OXTP with EC_{50} values higher than 4 mg/L (Table 4). Indeed, no substantial decrease in terms of I%I could be appreciated in the 0.0004-4 mg/L concentration range, and as consequence, IS values for each concentration remained very low, reaching a maximum of 27.5 % and 23.8 % respectively at 4 mg/L.

Table 4: Disease severity (I%I) on the untreated control, sporulation inhibition (IS) at each OXTP concentrations (0.0004-4 mg/L), and EC_{50} values obtained from sensitivity tests carried out on resistant strains Pv-16.1 and Pv-16-2 isolated from the Pv-16 sample during the experimental activities.

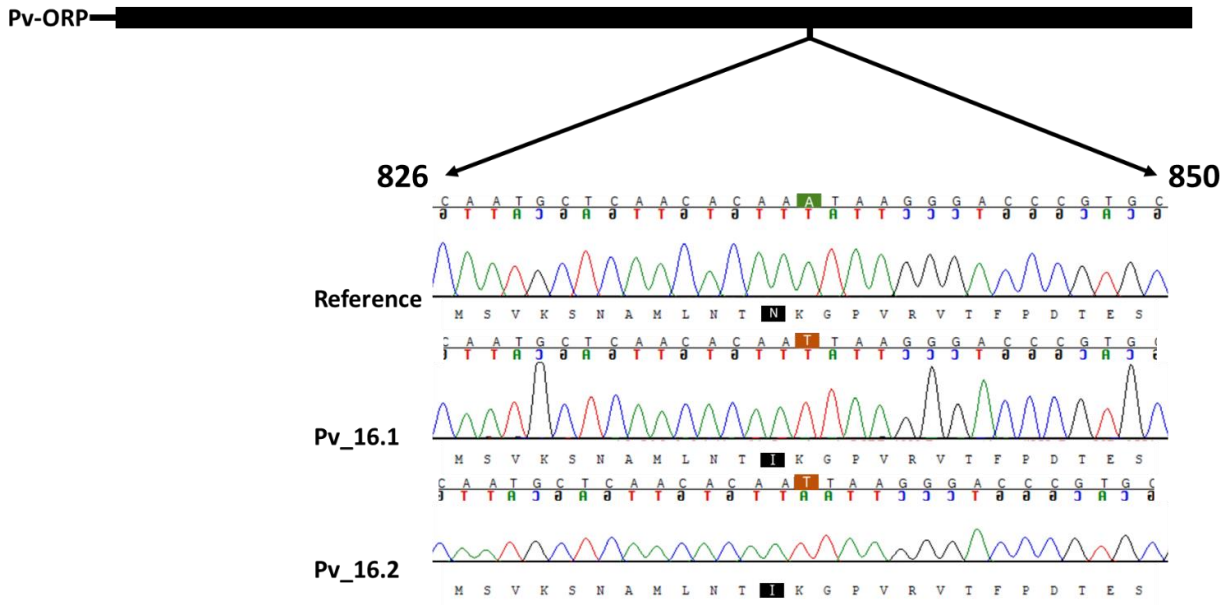
Sample code	I%I (%)	IS (%)					EC_{50} (mg/L)
	0	0.0004	0.004	0.04	0.4	4	
Pv-16.1	95.2	2.5	15.0	15.0	12.5	27.5	> 4
Pv-16.2	92.9	4.8	4.8	13.5	13.5	23.8	> 4

3.2 Molecular characterization of resistant isolates

Given the resistant phenotype detected in the sensitivity tests performed on the two isolates Pv-16.1 and Pv-16.2, DNA samples were collected from these samples and the coding region of the *PvORP1* gene was analyzed and compared on genomic DNA with 24 reference sensitive isolates tested as described above ($EC_{50} < 4 \times 10^{-3}$ mg/L) belonging to the DISAA collection and never exposed to OXTP. Analysis of *PvORP1* gene sequences in those two isolates revealed 5 single

nucleotide polymorphisms (SNPs), one of which changing the amino acid sequence of the PvORP1 proteins at position 37 (N37I) (Figure 3). This SNP is associated with a substitution of the nucleic acid adenine (A) with thymine (T) and leads to a codon change from AAT, encoding asparagine (N) in sensitive isolates, to ATT, encoding an isoleucine (I) residue in isolates expressing a resistant phenotype.

Figure 3: Sanger sequencing chromatogram of 826-850 nt region (arrows) of *PVORP1* of sensitive controls (reference) and resistant isolates Pv-16.1 and Pv-16.2. The amino acid encoded by codon 37 is marked using black squares. Upper sequences represent the coding strand and the lower sequence is the complement.



4. Discussion and conclusions

Since nowadays, the costs of research and development for new molecules have escalated (Russel, 2004), resistance development assumes a significant risk. The key element in detecting it before its spreading becomes meaningful is to know the sensitivity baseline for the considered fungus/fungicide combination. Only with this important information is it possible to observe if the fungicide response is changing towards resistance and preserve the efficacy of all the chemical classes available (Hobbelen et al., 2014).

In this study, the new molecule OXTP showed excellent activity against Italian *P. viticola* field populations. This is not surprising considering that this active substance has never been employed for downy mildew control in 50% of the sampled vineyards before 2019 (Pv-01, Pv-02, Pv-05, Pv-06, Pv-10, Pv-11, Pv-13, Pv-14, Pv-15, Pv-18, Pv-19, Pv-20, Pv-28, Pv-29). The sensitivity profiles obtained from these latter samples represent an accurate and heterogeneous Italian baseline sensitivity to OXTP, whereas the data obtained from populations sampled from vineyards treated with a variable number of OXTP applications, provide a more global vision of the resistance status in Italy on one hand, and represent a possible resistance evolutionary scenario after a single

growing season (Pv-03, Pv-04, Pv-07, Pv-08, Pv-09, Pv-12, Pv-16, Pv-17, Pv-21, Pv-22, Pv-23, Pv-24, Pv-25 and Pv-27) or more longer periods (Pv-26) on the other.

Despite the general high level of sensitivity, for two of the 29 populations tested (Pv-24 and Pv-26) the EC_{50} values obtained were over the maximum tested concentration of 4 x 10^{-1} mg/L of OXTP, which was tentatively considered the discriminatory dose for the identification of resistant isolates according to the results obtained from sensitivity tests. Indeed, less than 11% of the tested *P. viticola* populations (Pv-16, Pv-24 and Pv-26) were able to sporulate at this concentration, confirming this threshold as indicative of presence of OXTP resistance. This hypothesis is also supported by the RFs of the two populations previously mentioned, which were calculated on an average EC_{50} value of all the sensitive isolates as proposed by Mair et al. (2016) and Weber and Hahn (2011). RFs > 10 are usually indicative of resistance (Russel, 2004), and these two populations with RFs of 85.7 or more, could be therefore considered highly resistant to OXTP. In this context, it is interesting to note that the maximum value of EC_{50} was obtained from the population treated for 5 consecutive years with OXTP before 2019 (Pv-26). Despite the two following and consecutive growing seasons (2019 and 2020) in which this vineyard was not subjected to any spray program, the resistance level detected was very high. A possible explanation may be found in a lack of fitness penalties for the resistant strains present within this population, which could allow them to propagate over time. This assumption is also supported by I%I values of this sample (I%I=78.6% on the untreated control) which is comparable to those of sensitive isolates, and which does not undergo substantial decreases in terms of percentage until the last OXTP concentration (I%I= 42.3% at 4 x 10^{-1}mg/L). However, this type of test on sporangia does not allow to fully understand the percentage of resistant strains in the population. As a contradiction, it should be noted that for populations Pv-24 and Pv-26 it was not possible to successfully isolate stable single strains from survivors of sensitivity test in order to characterize the resistance mechanism with molecular tools. Further investigations are needed to better understand if there are any possible fitness costs associated with this partial tolerance to OXTP that could prevent the spread of *P. viticola* resistant strains in field conditions. To our knowledge, there are no data in the literature on this aspect for *P. viticola* to serve as points of reference, however studies on other oomycetes suggest that the survival potential of OXTP-resistant mutants in the field might be reduced (Bittner et al., 2017; Mboup et al., 2022; Miao et al., 2016 and 2018).

The *P. viticola* strains bearing the mutated *PvORP1* allele (*PvORP1*-37I) isolated from sample Pv-16 exhibited reduced levels of sensitivity to OXTP, while reference isolates carrying the wild-type allele (PvORP1-37N) expressed a sensitive phenotype and were unable to grow at 4 x 10^{-1} mg/ml of OXTP. This suggests a correlation between the presence of this mutation and the resistant phenotype found in sensitivity tests. This aminoacidic substitution has already been reported in *P. viticola* Italian field populations by Mboup and collaborators (Mboup et al., 2022) as conferring resistance. Despite the authors were not able to determine the level of resistance of this SNP in *P. viticola*, our data tend to confirm their hypothesis of a mutation conferring high resistance levels. For the two resistant strains (Pv-16.1 and Pv-16.2) in leaf-discs sensitivity tests we found very low IS values, until the maximum fungicide concentration tested (IS=27.5% and 23.8 % at 4 mg/L, respectively), suggesting that individuals carrying the N37I mutation could easily survive and infect *V. vinifera* species in presence of OXTP. Moreover, other point mutations determining amino acid substitutions at position N837 have been reported to confer high levels of resistance to fluoxapiprolin (same chemical class as OXTP) in other oomycetes (Miao et al., 2021). This further corroborates the hypothesis that the residue at this position can significantly affect biological

activity of OSBP inhibitors. Further study is needed to confirm possible fitness costs linked to *PvORP1*-37I allele, and disease management strategy must be carefully planned, taking into consideration the possible spread of this mutation in field conditions.

Results from this work indicate an excellent activity of OXTP against *P. viticola* populations never exposed to this fungicide. At the same time, in some situations in which the pathogen was exposed even for short periods and for few applications to the fungicide, low sensitivity was recorded. Similar observations have been made investigating OXTP efficacy in *P. viticola* and in other oomycetes species (Mboup et al., 2022; Miao et al., 2021; Miao et al., 2016).

Given the difficulty and cost of registration of single-site fungicides such as OXTP, and the imperative of preserving their effectiveness as long as possible, we conclude that in the case of high-risk of resistance pathogens such as *P. viticola*, prolonged applications of this molecule in the same location should be avoided. We suggest to include OXTP in spray programs in mixture and/or alternation with partners possessing a different mode of action, as reported by good anti-resistance management practices (Brent & Hollomon, 2007; FRAC, 2020), and to pay particular attention when using this fungicide in areas characterized by high disease pressures levels which could be considered to be more susceptible to the risk of resistance spreading.

5. Bibliography

Andreassi, J. L., Gutteridge, S., Pember, S., & Sweigard, J. A. (2013). *Detection and Screening Method and Materials Useful in Performance Thereof* (Patent No. WO13/009971).

Bittner, R. J., Sweigard, J. A., & Mila, A. L. (2017). Assessing the resistance potential of *Phytophthora nicotianae*, the causal agent of black shank of tobacco, to oxathiopropalin with laboratory mutants. *Crop Protection*, *102*, 63–71. https://doi.org/https://doi.org/10.1016/j.cropro.2017.08.002

Brent, K. J., & Hollomon, D. W. (2007). Fungicide resistance in crop pathogens. How can it be managed. In *FRAC Monograph No. 1* (2nd ed.). CropLife International.

Cohen, Y. (2015). The novel oomycide oxathiapiprolin inhibits all stages in the asexual life cycle of *Pseudoperonospora cubensis* - Causal agent of cucurbit downy mildew. *PLoS ONE*, *10*(10), 1–22. https://doi.org/10.1371/journal.pone.0140015

FRAC. (2020). *Resistance overview*. www.frac.info

FRAC. (2021). *List of monitoring methods*. www.frac.info/monitoring-methods

Hobbelen, P. H. F., Paveley, N. D., & van den Bosch, F. (2014). The emergence of resistance to fungicides. *PLOS ONE*, *9*(3), 1–14. https://doi.org/10.1371/journal.pone.0091910

Ji, P., & Csinos, A. S. (2015). Effect of oxathiapiprolin on asexual life stages of *Phytophthora capsici* and disease development on vegetables. *Annals of Applied Biology*, *166*(2), 229–235. https://doi.org/https://doi.org/10.1111/aab.12176

Ji, P., Csinos, A. S., Hickman, L. L., & Hargett, U. (2014). Efficacy and application methods of oxathiapiprolin for management of black shank on tobacco. *Plant Disease*, *98*(11), 1551–1554. https://doi.org/10.1094/PDIS-02-14-0172-RE

Lafon, R., & Clerjeau, M. (1988). Downy mildew. In R. C. Pearson & A. C. Goheen (Eds.), *Compendium of grape diseases* (pp. 11–13). APS Press.

Ma, Z., & Michailides, T. J. (2005). Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop Protection*, *24*(10), 853–863. https://doi.org/https://doi.org/10.1016/j.cropro.2005.01.011

Mair, W. J., Deng, W., Mullins, J. G. L., West, S., Wang, P., Besharat, N., Ellwood, S. R., Oliver, R. P., & Lopez-Ruiz, F. J. (2016). Demethylase inhibitor fungicide resistance in *Pyrenophora teres* f. sp. *teres* associated with target site modification and inducible overexpression of *Cyp51. Frontiers in Microbiology*, *7*, 1279. https://doi.org/10.3389/fmicb.2016.01279

Massi, F., Torriani, S. F. F., Borghi, L., & Toffolatti, S. L. (2021). Fungicide resistance evolution and detection in plant pathogens: *Plasmopara viticola* as a case study. *Microorganisms*, *9*(1), 1–18. https://doi.org/10.3390/microorganisms9010119

Mboup, M. K., Sweigard, J. W., Carroll, A., Jaworska, G., & Genet, J. (2022). Genetic mechanism,

baseline sensitivity and risk of resistance to oxathiapiprolin in oomycetes. *Pest Management Science*, *July*. https://doi.org/10.1002/ps.6700

Miao, J., Cai, M., Dong, X., Liu, L., Lin, D., Zhang, C., Pang, Z., & Liu, X. (2016). Resistance assessment for oxathiapiprolin in *Phytophthora capsici* and the detection of a point mutation (G769W) in PcORP1 that confers resistance. *Frontiers in Microbiology*, *7*, 615. https://doi.org/10.3389/fmicb.2016.00615

Miao, J., Chi, Y., Lin, D., Tyler, B. M., & Liu, X. (2018). Mutations in ORP1 conferring oxathiapiprolin resistance confirmed by genome editing using CRISPR/Cas9 in Phytophthora capsici and P. sojae. *Phytopathology*, *108*(12), 1412–1419. https://doi.org/10.1094/PHYTO-01-18-0010-R

Miao, J., Dong, X., Lin, D., Wang, Q., Liu, P., Chen, F., Du, Y., & Liu, X. (2016). Activity of the novel fungicide oxathiapiprolin against plant-pathogenic oomycetes. *Pest Management Science*, *72*(8), 1572–1577. https://doi.org/https://doi.org/10.1002/ps.4189

Miao, J., Li, C., Liu, X., Zhang, X., Li, G., Xu, W., Zhang, C., & Liu, X. (2021). Activity and resistance-related point mutations in target protein PcORP1 of fluoxapiprolin in *Phytophthora capsici. Journal of Agricultural and Food Chemistry*, *69*(13), 3827–3835. https://doi.org/10.1021/acs.jafc.0c05119

Pasteris, R. J., Hanagan, M. A., Bisaha, J. J., Finkelstein, B. L., Hoffman, L. E., Gregory, V., Andreassi, J. L., Sweigard, J. A., Klyashchitsky, B. A., Henry, Y. T., & Berger, R. A. (2016). Discovery of oxathiapiprolin, a new oomycete fungicide that targets an oxysterol binding protein. *Bioorganic & Medicinal Chemistry*, *24*(3), 354–361. https://doi.org/10.1016/j.bmc.2015.07.064

Raychaudhuri, S., & Prinz, W. A. (2010). The diverse functions of oxysterol-binding proteins. *Annual Review of Cell and Developmental Biology*, *26*, 157–177. https://doi.org/10.1146/annurev.cellbio.042308.113334

Russel, P. (2004). Sensitivity baselines in fungicide resistance research and management. In *FRAC Monograph 3*. CropLife International.

Toffolatti, S. L., Russo, G., Campia, P., Bianco, P. A., Borsa, P., Coatti, M., Torriani, S. F., & Sierotzki, H. (2018). A time-course investigation of resistance to the carboxylic acid amide mandipropamid in field populations of *Plasmopara viticola* treated with anti-resistance strategies. *Pest Management Science*, *74*(12), 2822—2834. https://doi.org/10.1002/ps.5072

Weber, R. W. S., & Hahn, M. (2011). A rapid and simple method for determining fungicide resistance in *Botrytis. Journal of Plant Diseases and Protection*, *118*(1), 17–25. http://www.jstor.org/stable/43229155

Characterization of the *Plasmopara viticola* sporangia suspension composition and evaluation of its infection efficiency and germination dynamics by means of flow cytometry and fluorescence activated cell sorting

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Abstract:

Plasmopara viticola, the causal agent of grapevine downy mildew, is one of the most important pathogens in viticulture world panorama. *P. viticola* is a polycyclic pathogen, able to carry out numerous cycles of infection during a single vegetative grapevine season, both as a result of sexual and of asexual reproduction. Studying the infections of *P. viticola,* depending on the life stages of the pathogen under investigation, more quantitative or qualitative data can be obtained according to the testing methods available. This creates a great inhomogeneity in the overall investigation of infection process of this oomycete.

Flow cytometry is an automated technique of multi-parametric analysis based on laser as a light source to produce signals exploitable in analysis of physical and chemical characteristics of a single cell within a population. Fluorescence Activated Cell Sorting is a specialized form of flow cytometry that provides a method for recognizing and sorting single cells within a heterogeneous cell suspension.

In this study, a technique based on this technology was developed to investigate secondary infection cycles of *P. viticola,* which since today have been studied only from a qualitative point of view. In detail, the composition, the infection efficiency and the infection dynamics of a sporangia suspension of this pathogen were evaluated. It was possible to recognize the presence of different structures within the spore suspension, including viable sporangia. The latter have been isolated and correctly sorted within the developed test, which allowed detecting new interesting parameters currently not investigated.

1. Introduction

Grapevine downy mildew, caused by the phytopathogenic oomycete *Plasmopara viticola* (Berk. *et* Curtis) Berl. & De Toni, is one of the major threats to grapevine production worldwide. Indeed, severe disease epidemics caused by this oomycete are often associated with consistent quantitative and qualitative yield losses (Lafon & Clerjeau, 1988).

P. viticola is an obligate, biotrophic pathogen, able to undergo numerous infection cycles during a single grapevine-growing season (Gessler et al., 2011). In autumn, the pathogen develops overwintering structures differentiated by sexual reproduction (oospores), through which it survives the winter period embedded in dead leaves on the vineyard floor (Gessler et al., 2011; Rossi & Caffi, 2007; Vercesi et al., 2002). In spring, at the occurrence of favorable climatic conditions (Maddalena et al., 2021), oospores germinate typically producing sporangia that, in turn, produce zoospores. In presence of receptive tissues of grapevines, leaves are infected by zoospores through splashing by rain, originating the primary infections through stomata penetration (Hill, 1998; Vercesi et al., 1999). The pathogen develops an intercellular mycelium with haustoria (feeding structures) and, in high humidity conditions, differentiates sporangiophores that emerge from stomata and produce sporangia, that will originate secondary infections cycles through the emission of new zoospores (Gessler et al., 2011).

Oospores are considered to play a main role in triggering the epidemic in the early grapevine season, providing the inoculum for primary infections, while the subsequent stages (secondary infections) are attributed to the inoculum generated by asexual reproduction through the differentiation of sporangia (Lafon & Clerjeau, 1988; Vercesi et al., 2002).

Despite there are many testing methods to investigate oospores germination process and oospores infection efficiency (Lehoczky, 1965; Maddalena et al., 2021; Si Ammour et al., 2020; Toffolatti et al., 2007, 2018; Toffolatti et al., 2011; Vercesi et al., 2002; Vercesi et al., 2010), to the best of our knowledge no studies have been conducted to evaluate the infection potential associated with the sporangia inoculum in vineyards. Up to date, testing methodologies on sporangia germination are limited because they can provide only a qualitative description of infection process, and it is not possible to obtain a precise estimation of the percentage of sporangia able to positively infect grapevine plants in the population tested (Corio-Costet, 2015; Massi et al., 2021). This is particularly problematic because it makes the data obtainable by investigating the primary and secondary infections cycles inhomogeneous, and creates a gap of information.

Fluorescence Activated Cell Sorting (FACS) technology is a technique to purify specific cell populations based on phenotypes detected by flow cytometry (FCM) which can represent an interesting opportunity to improve information on sporangia infection process (D'hondt et al., 2011; Ishii et al., 2010; Shapiro, 2005). Briefly, FCM is a technology able to provide rapid multi-parametric analysis of single cells in solution using lasers as light sources to produce both scattered and fluorescent light signals that are read by detectors such as photodiodes or photomultiplier tubes (Winson & Davey, 2000). These signals are converted into electronic signals that are analyzed by a computer. FACS implementation provides a method for sorting a

heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell (Czechowska et al., 2008). Considering the continuous advancement of these technologies, the use of FCM rapid protocols to quantify single cells, such as fungal spores, can be set up. Indeed, FCM allows the researcher to monitor different useful parameters to distinguish between living and dead cells such as membrane permeability, efflux pumps or enzymatic activity and loss of membrane potential (Joux and Lebaron, 2000).

Implementations of FCM technologies in fungal plant pathogens investigations could be a useful and innovative approach, which, in the case of *P. viticola,* could permit to bridge the gap of information obtainable by studying the infection process in the different life stages of pathogens as mentioned above. This could represents a big step forward in the study of grapevine downy mildew, allowing to study the secondary infection cycles of the pathogen with greater completeness and providing quantitative data. To date, no applications of FMC on infection efficiency screening in plant pathogens are available. To the best of our knowledge, the only implementations of FCM have been carried out on the related oomycete species *Phytophthora infestans* (Mont.) de Bary (Catal et al., 2010; Day et al., 2002). In particular, Day and collaborators used FCM to discriminate *P. infestans* sporangia from other airborne biological particles by using light scatter parameters and fluorescent staining.

Furthermore, a unique study to determine the *P. viticola* sporangia viability exposed to chlorine dioxide using fluorescent dyes was performed by Sergeeva and collaborators (Sergeeva et al., 2002).

The aim of the present work was to characterize the sporangia suspension composition in *P. viticola* and to evaluate its infection efficiency and germination dynamics. This was accomplished by developing a FACS mediated single-sporangia infection assay. In this context, infection efficiency is defined as the ratio of sporangia able to infect grapevine leaf tissues.

2. MATERIALS AND METHODS

2.1 *P. viticola* material

A *P. viticola* monosporangial isolate, belonging to the collection of the Department of Agricultural and Environmental Sciences (Disaa, University of Milan, Italy) was used in this study. This *P. viticola* strain, designated CAS, has been isolated in 2016 as a part of an Italian downy mildew genetic study (Maddalena et al., 2020; Sargolzaei et al., 2020) from a field population of north-eastern Italy (Casarsa della Delizia, province of Pordenone). From its isolation to the beginning of the experimental activity, the isolate has been maintained through weekly propagation on detached grapevine leaves (cv Pinot noir) (Toffolatti et al., 2018). Large numbers of sporangia for experiments were obtained by massively propagating the strain on 100 leaves.

The sporangia suspension was obtained by collecting the sporangia present on grapevine leaves, 7 days after inoculation in 5 mL of Phosphate Buffered Saline (PBS pH 7.4, Merk Life Science S.r.l., Milan, Italy). PBS was filtered through a 0.2-µm pore size PVDF membrane filter and sterilized in an autoclave (121 °C, 20 min). Sporangia concentration was determined by using Kova chambers (Kova International, Garden Grove, CA, USA) under microscope (Zeiss Primo Vert, Carl Zeiss Microscopy, NY, USA) and adjusted to a final concentration of $1x10^7$ sporangia/mL. The sporangia suspension was filtered through a 30 µm-pore size strainers (Sysmex, Gorlitz, Germany) immediately before the flow cytometry and FACS sorting.

2.2 Sample staining and flow cytometry analyses

Flow cytometry analyses were performed on the Accuri C6 Plus (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometer equipped with blue (488 nm, 20 mW) laser. Forward scatter (FSC), side scatter (SSC) and red fluorescence (>670 nm in FL3 channel equipped with 670LP filter) signals were collected by acquiring 9,000 events. Either unstained sporangia were analyzed, or staining with 7.5 µM (final concentration) aqueous solution of Propidium Iodide (PI) was implemented.

To measure possible variations linked to sporangia metabolic state changing over time, data acquisition was performed 0.5 h after the sporangia sampling (HPSS) and repeated a second time 4.0 HPSS. During this period, the sporangia remained suspended at 22°C in PBS (pH 7.4). All parameters were collected as logarithmic signals, and data were analysed with the BD Accuri™ C6 Plus software ver. 1.0.23.1.).

2.3 Fluorescence activated cell sorting and microscopy

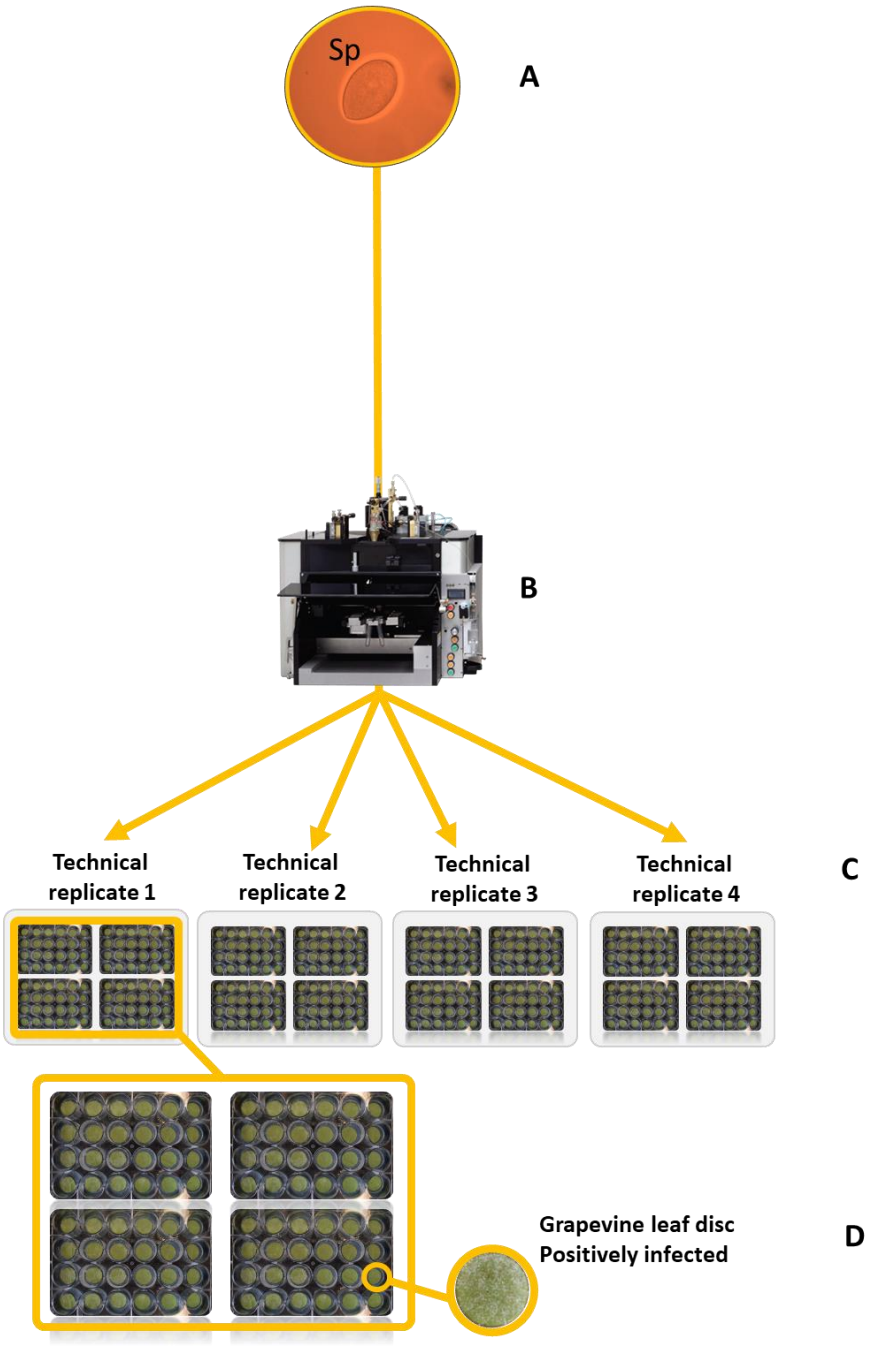
To characterize the composition of fresh and unstained *P. viticola* sporangia suspension, individual subpopulations detected during FCM analysis (Figure 1A) were isolated by single-cell fluorescence activated sorting. In detail, fresh and unstained sporangia suspension, was 2.5-fold diluted with a PBS buffer and subjected for sorting on the FACSJazz™ (BD Biosciences, Franklin Lakes, NJ, USA) cell sorter, equipped with a blue laser (488 nm, 80 mW). The instrument was triggered on forward scatter (FSC-H), and the sort gate was set firstly on forward scatter (FSC-H) versus side scatter (SSC-H) of 5 and 7 µm diameter uniform microspheres and further on side scatter (SSC-H) versus red fluorescence (FL3-H). The drop delay was calculated with AccuDrop™ beads (BD Biosciences) using BD FACS™ Sortware v1.2. The fixed sheath pressure used was 27 psi. The sample differential was set at 0.5 psi, and it was kept constant for the duration of the experiment. The drop drive frequency was 39.20 kHz.

Once sorting parameters were established, the subpopulations (SSC-H *vs* FL3-H) detected inside the sporangia suspensions were sorted using either the sort mode “1.0 drop Pure” into sterile 15 mL centrifuge tubes [subsequently observing an aliquot in a bright field under a light microscope (AX10 Axio Lab A1, Zeiss, Carl Zeiss Microscopy, NY, USA) at 100x magnification with immersion oil to assess the identity of the sorted structures] or the sort mode “1.0 drop Single” in case of sorting on grapevine leaf discs as reported below.

2.4 Infection efficiency assay

To assess the infection efficiency of the *P. viticola* asexual spores, a FACS mediated single-sporangia infection assay was designed. In detail, single sporangia were isolated and sorted through FACS on healthy grapevine leaf discs placed inside a 24-well microtiter plate. The experimental scheme of the assay is reported in Figure 1.

Figure 1: Schematic representation of the sporangia infection efficiency assay. Sporangia (Sp) present inside the suspension (A) were isolated by sorting (B) and individually inoculated on a leaf disc present within wells of a 24-multiwell plate (C). Each experimental repetition consisted of four 24-multiwell plates for a total of 96 leaf discs. From day 4 to 14, the presence of sporulation was visually assessed on each inoculated disc to confirm or not of the infection (D).



Four experimental replicates were performed, and for each of them, four 24-well microtiter plates (Falcon multi-well 24, Biosigma, Cona, Italy) were filled in with 1 mL of water-agar (0.5%, Agar bacto BD DIFCO; Becton Dickinson Italia, Milan, Italy) per well. Grapevine (*Vitis vinifera* L. cv Pinot noir) leaf discs (15 mm diameter) were excised with a cork borer and placed with their abaxial surface upwards in single wells, 24 hours before the inoculation. Immediately before the inoculation, the leaf discs were sprayed with sterile distilled water to reach 100% relative humidity inside the plate. An additional plate (not inoculated) was prepared for each replicate as a negative control.

Single *P. viticola* sporangia were sorted using the "1.0 drop Single" sort mode, and each leaf disc was inoculated with a drop containing a single sporangium.

To exclude damage of the sporangia suspensions during the sorting process, three healthy grapevine leaves (cv. Pinot noir) were placed with their abaxial surface up, in a Petri dish with moistened filter paper, and inoculated by spraying 1 mL of the corresponding sporangia suspension.

Immediately after the inoculation, all plates were incubated for 14 days in a climate chamber (22 °C, 10 µmol/m²s, 12/12 h light/darkness period, 70% relative humidity). Leaf discs were scored daily under microscope (Zeiss Primo Vert, Carl Zeiss Microscopy, NY, USA) from 4 to 14 days post inoculation (DAI) to check for the presence of sporulation, as confirmation of successful outcome of the infection process. In this context, infection efficiency was defined as the ratio of positively infected leaf discs over the total.

2.5 Sporangia infection dynamics

Because the single-sporangia assay reproduces in laboratory conditions the sporangia infection dynamics (SID) along the experimental timeframe of investigation of 14 days (*i.e.*: time function), such phenomenon can be investigated by fitting a Generalized Linear Mixed Model (GLMM) defined as follows:

$$g(y_{tij}) = \eta_{tij} = \beta_0 + \beta_{ti}t + \beta_j + \varepsilon_{ij}$$

where y_{tij} is the number of positively infected leaf disc out of the total number n_i of inoculated leaf discs (*i.e.*: the proportion of infecting sporangia) at time t for the i-th plate with i={plate1, plate2, plate3 … plate16} and at the j-th experiment with j={Experiment1, Experiment2,Experiment3, Experiment4}; η_{tij} is the linear predictor expressed according to the Probit link function $g(\bullet) = \Phi(\bullet)$; $\beta_0=b_0+u_{0i}$ is the intercept, where b_0 is the intercept's fixed term and u_{0i} is the intercept's random component which represents the plate-dependent effect arising from the random inclusion of any i-th plate in the j-th experiment; $\beta_{ti}=b_t+\tau_i$ is the slope for the time effect on the i-th plate's SID, where b_t is the fixed slope's component representing the sporangia infection general trend in time and τ_i is the random plate-dependent slope's component accounting for the plate's effect on SID; t is the time expressed as DAI, which is the same for any i-th plate and j-th experiment, as all of the plates were inoculated and incubated on the same day; β_j is the fixed experiment's effect and ε_{ij} is the error term. The variance heterogeneity is managed by setting n_i as weights during the GLMM

fitting procedure. This GLMM parameterization allows one to compute both a general $t_{50}=-b_0/b_t$ (named here as the GLMM t_{50}) and a plate-specific $t_{50}^i=-\beta_0/\beta_t$ at one time, according to the equation suggested by Faraway (2016) Such index is useful to summarize the spore germination dynamics, such as in the case of *P. viticola* oospores (Maddalena et al., 2021). Thus, assuming that all of the sporangia used in this trial were collected from the same population and the GLMM described above is really representative of the SID, (*i.e.*: the GLMM's fitted data values approach the observed ones according to the pseudo-R^2 computed via Observed vs Simulated simple linear regression after (Piñeiro et al., 2008), the single-sporangia assay proposed here can be considered reliable if: i) the experiment fixed effect β_j is not significantly different from 0 for $\alpha=0.05$ and the simulated data don't fall out of the 95% Probit confidence limits (Dorai-Raj, 2014) computed for the overall observed infecting sporangia at the time t; ii) the t_{50}^i expected value is not significantly different from general t_{50} for $\alpha=0.05$. The former condition is readily assessed by the computing the Wald's χ^2 test (Type II solution) for the GLMM parameters (Fox, 2015) and by comparing simulated data to 95% Probit confidence limits cited above; the latter condition is assessed by comparing the general t_{50} value (*i.e.*: the GLMM t_{50}) to the bootstrap t_{50} and its 95% confidence limits. The bootstrap t_{50} is computed by resampling 10^4 times by bootstrap method (Efron et al., 1993) from the set of t_{50}^i and then obtaining its expected value as a mean of the values arising from 10^4 simulations. The bootstrap t_{50} 95% is directly computed by extracting the 2.5^{th} and the 97.5^{th} quantile from the bootstrap distribution.

GLMM Model as described above is fitted by glmer() function implemented in lme4 R 3.4.3. statistical packages, whereas its Type II solution is computed by Anova() function implemented in car R 3.4.3. statistical packages. The 95% Probit confidence limits (Dorai-Raj, 2014) computed for the overall observed SID are computed by binom.probit() function implemented in binom R 3.4.3. statistical packages, whilst the bootstrap simulations and bootstrap 95% confidence limits are obtained by bootstrap() function implemented in bootstrap R 3.4.3 statistical packages and quantile() function implemented in stats R 3.4.3. statistical packages.

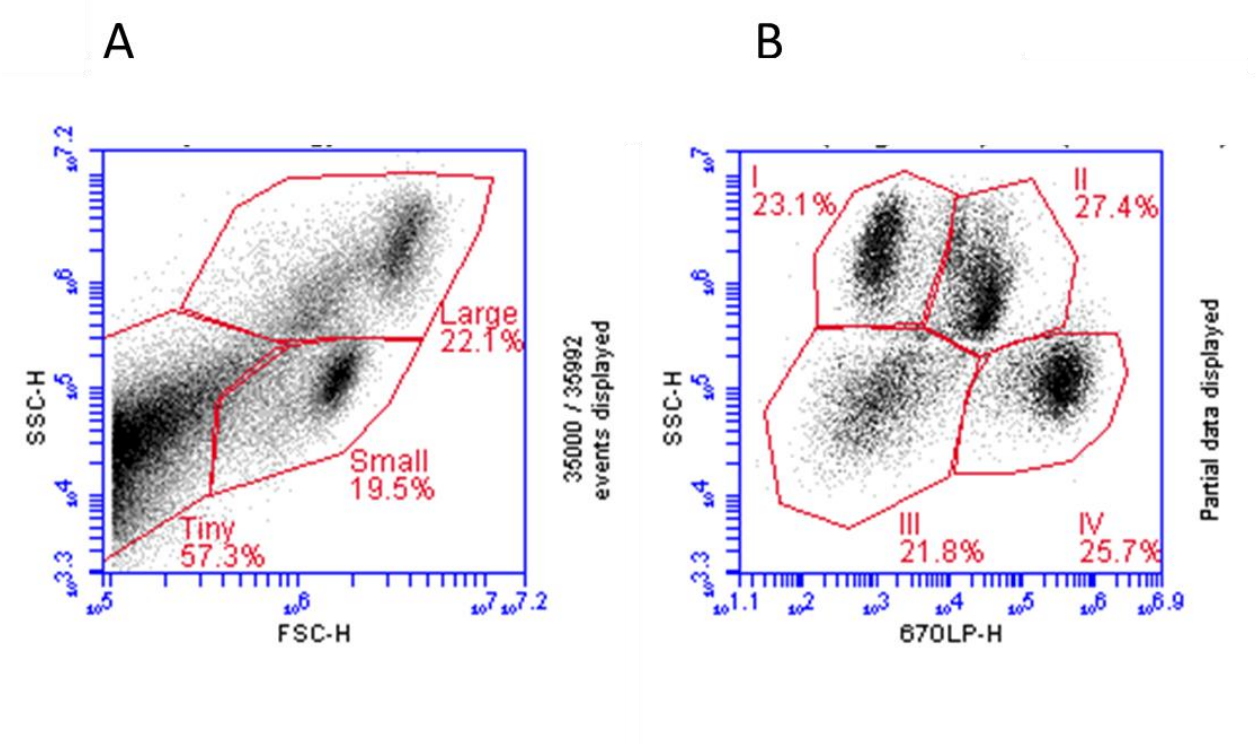
3. RESULTS

3.1. Characterization of the *Plasmopara viticola* sporangia suspension composition

The sporangia suspension was obtained by collecting from grapevine leaves, as described in Materials and Methods. With the aim to characterize the *Plasmopara viticola* sporangia suspension composition, it was subjected to the flow cytometry analysis. Data were firstly gated by using polygonal gates drawn on plots of FSC versus SSC. By using a combination of measurements of FSC and SSC *P. viticola* sporangia suspension could be divided into three subpopulations denominated "Large" (> 0.7 µm), "Small" (0.5 – 0.7 µm) and "Tiny" (< 0.5 µm) (Figure 2A). "Large" and "Small" were those with size comparable to *P. infestans* sporangia as reported by Day and coworkers (Day et al., 2002). Other smaller particles were detected (gated "Tiny"), but they were attributed to the sample background debris. This last operation is particularly important for sporangia of pathogens such as *P. viticola* which has been isolated from leaves, and which therefore can be mixed with a considerable number of foreign particles of no

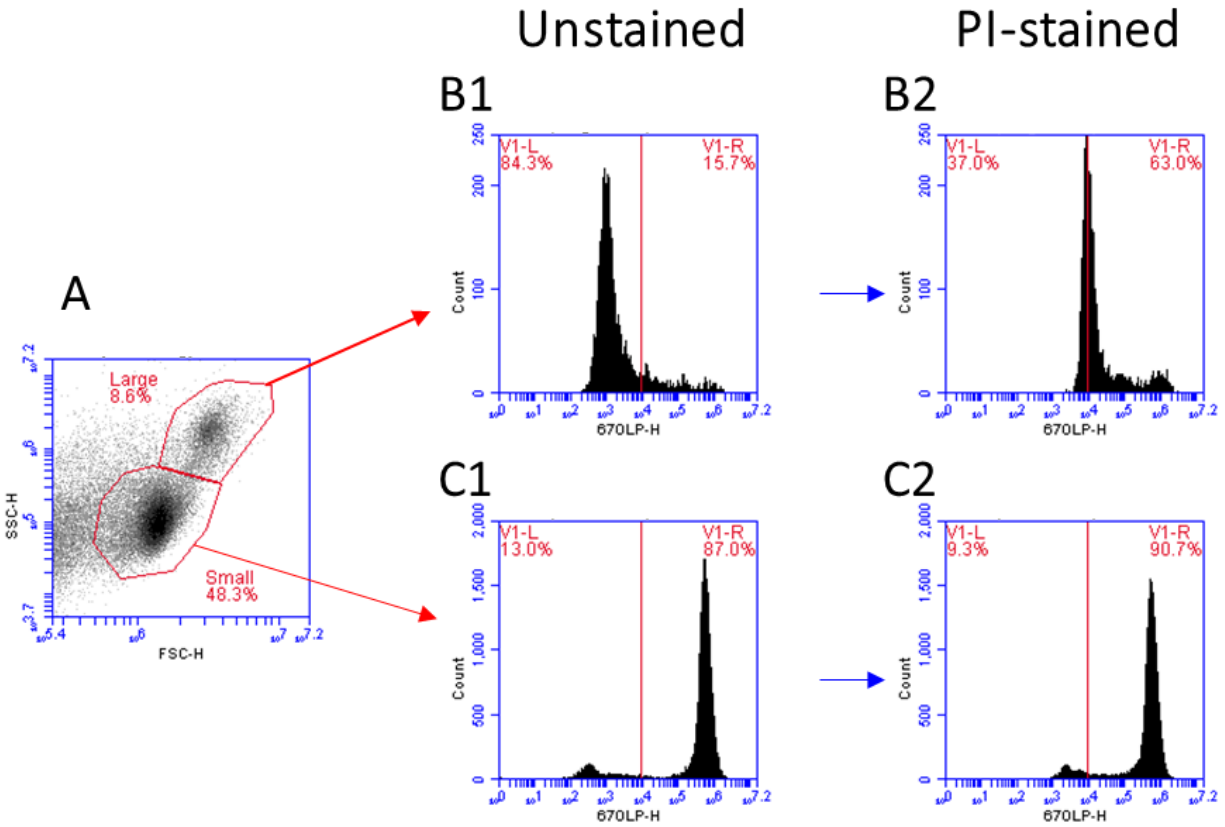
relevant interest. Secondary gates were subsequently drawn on SSC-H versus FL3-670LP cytograms, to include only particles the FSC and SSC of which matched that of the sporangia. Therefore, four subpopulations were clearly distinguishable (Figure 2B; I; II; III; IV).

Figure 2: (A) FSC-H vs SSC-H dots plot of a *P. viticola* sporangia suspension in which are visible two groups of particles (named "Small" and "Large") compatible with the assumed size of the sporangia. (B) SSC-H vs 670 LP-H (long pass) filter dots plot of "Small" and "Large" groups detected, within which four distinct subpopulations are visible (I, II, III and IV).



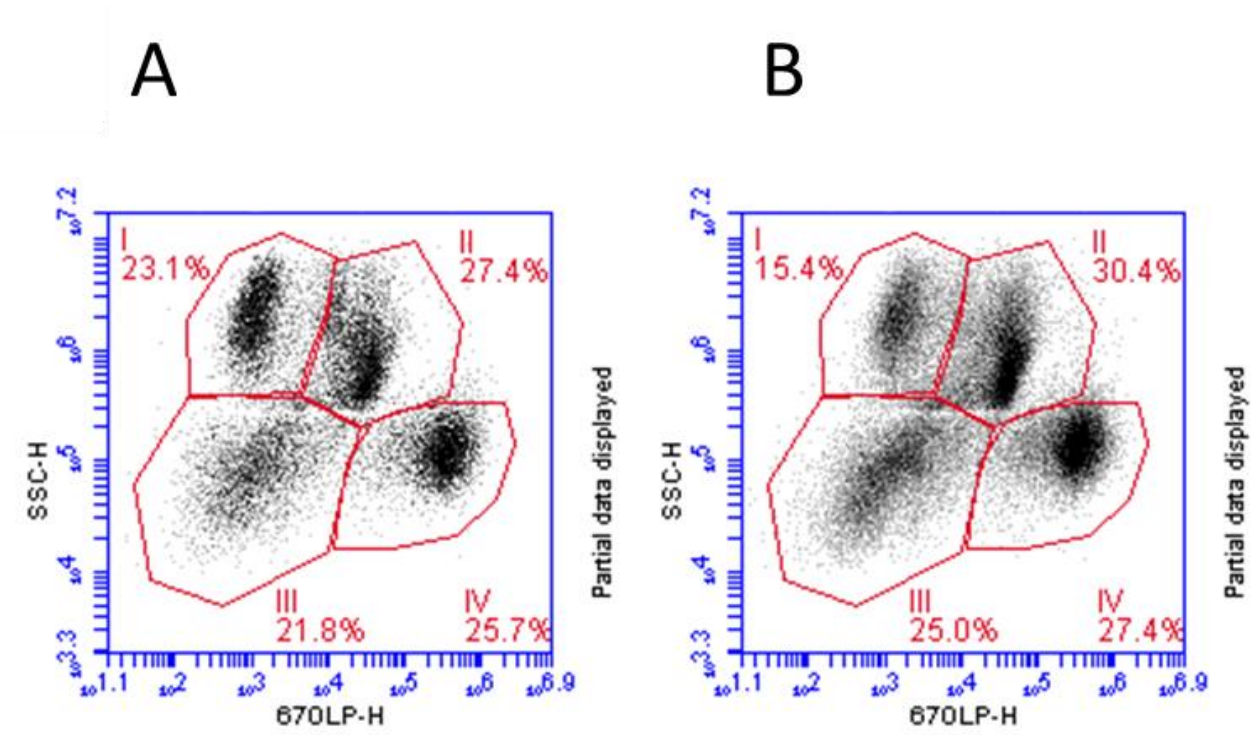
The gated biological particles (especially in the "Small" population) emitted a strong red autofluorescence, since no substantial shifts after staining were detectable in FCM data acquisition (Figure 3). Considering that red staining with PI is based on a nucleic acid stain able to enter only in the cells with significant membrane damage, it was not possible to distinguish between damaged cells stained with PI and intact cells exhibiting natural red autofluorescence.

Figure 3: (A) FSC-H vs SSC-H dots plot of a *P. viticola* sporangia suspension, and red fluorescence (>670 nm) count for "Large" (B) and "Small" (C) subpopulations unstained (1) and PI-stained (2). For "Small" population no substantial shifts are detectable after staining.



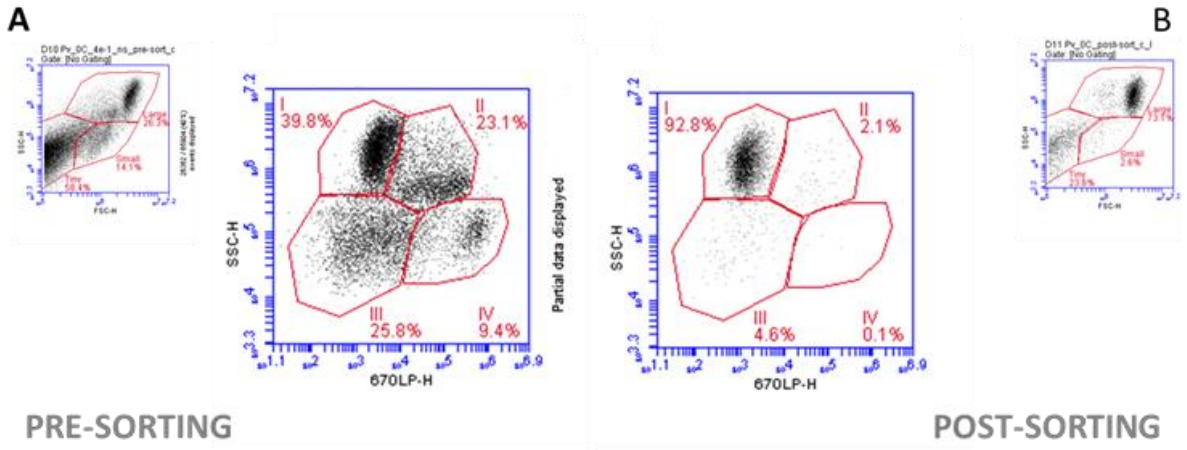
To assess light variations in particle size and/or metabolic state over time of the particles in the sporangia suspension, the same sample was reanalyzed with FCM after 4 HPSS, keeping the sporangia at 22 °C and using the same parameters previously mentioned. SSC-H and red fluorescence parameters were plotted, and shifts have been recorded in the percentage composition among the four subpopulations detected (Figure 4). In particular, it has been recorded a strong reduction in percentage of subpopulation I (from 23.1% to 15.4%) in favor of subpopulation II (27.4% to 30.4%). The composition of the other two subpopulations has also changed, albeit to a lesser extent.

Figure 4: SSC-H vs Red fluorescence (670 nm) dots plots of the *P. viticola* four subpopulations (I, II, III and IV) composing the sporangia suspension after (A) 0.5 HPSS in PBS (pH 7.4) and 4 HPSS in PBS (B).



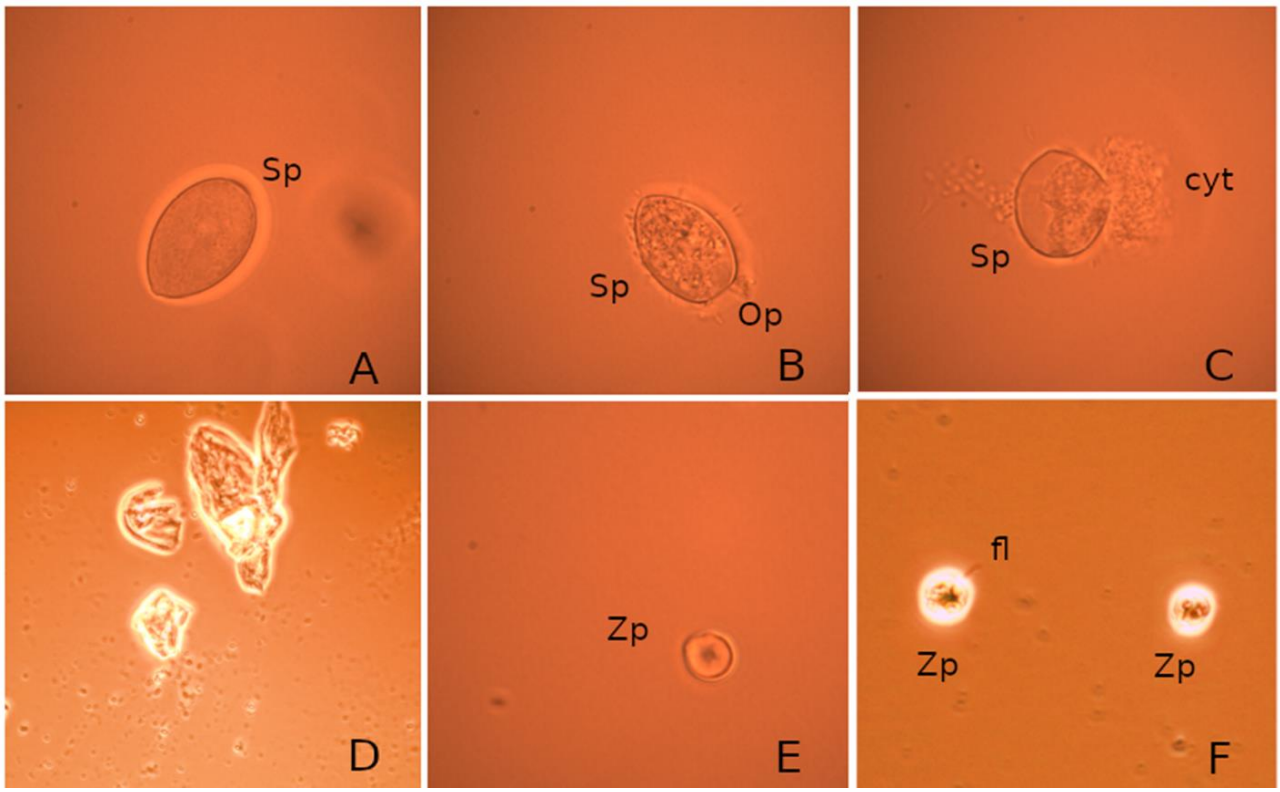
Considering the results obtained in the FCS analysis, FACS was used to isolate and sort on microscope slides the four subpopulations detected, to identify the different *P. viticola* structures under bright field microscope. FACS proved to be capable of accurately isolating and sorting all the 4 subpopulations identified (Figure 5A), as confirmed by the repetition of FCM analysis on an aliquot of the sorted sample (Figure 5B).

Figure 5: SSC-H vs Red-fluorescence dots plots of the *P. viticola* four subpopulations (I, II, III and IV) (A), and SSC-H vs Red-fluorescence dots plots after sorting of subpopulation (B).



After this initial result, it was possible to proceed with the characterization under microscope of the subpopulations. The visual study of the detected structures revealed that the subpopulation I, was entirely composed by *P. viticola* sporangia (Figure 6A), cells appeared with an intact cell wall and full of cytoplasm. The population II was mainly composed of degenerated or damaged sporangia (Figure 6B-C): in some cases, the cells displayed open opercula and exhibited a granular cytoplasm (Figure 6B), in other cases the cells appeared with a broken cell wall and a consistent loss of cytoplasm (Figure 6C). The population III was composed by debris of undefined nature, composed of plant residues deriving from the collection procedures of the spores or empty sporangia (Figure 6D). The population IV was composed both of encysted (Figure 7E) and swimming (Figure 6F) zoospores whose movement was clearly visible at the microscope.

Figure 6: Intact *P. viticola* sporangia isolated from population I (A). Sporangia with open operculum and granular cytoplasm (B). Emptying sporangia with broken cell wall and cytoplasm loss isolated in population II (C). Undefined debris isolated from population III (D). *P. viticola* zoospore encysted (E) and biflagellate swimming zoospores (F) isolated in population IV. Op: opercolum, Sp: sporangia, Zp: zoospore, fl: flagella, cyt: cytoplasm loss. All the pictures were taken at 100x magnification with immersion oil.



3.2 Evaluation of *P. viticola* infection efficiency and sporangia germination dynamics

Considering the results of the FACS mediated characterization of sporangia suspensions, and the clear identification of intact sporangia within subpopulation I, this group of homogeneous particles was selected to be sorted in the 24-well microtiter plates containing grapevine leaf discs. Indeed, as the aim of this experiment was the evaluation of sporangia infection efficiency, it would not have been productive to inoculate emptying sporangia with broken cell wall, debris or biflagellate zoospores. Although the latter are theoretically capable of generating infections by their own and

may also can be sporadically found inside the emptying sporangia or debris, the reproductive structure within which they form (the sporangium) contains numerous zoospores with the same genetic heritage, considerably increasing the chances of positive infection if inoculated. Therefore, it was reasonably chosen to investigate subpopulation I for infection assay.

Results obtained in the four experimental repetitions are reported in Table 1. The cumulative number of single sporangia able to infect the inoculated leaf disks at 14 days for the 24-well microtiter plates, ranged from a minimum of 0 in plate number 4.2 to a maximum of 4 obtained in plate number 3.1, 3.2 and 4.4 (Table 1). The total number of infected leaf discs obtained for each of the four experimental repetitions is respectively 6, 6, 10 and 10. As consequence, considering infection efficiency (IE %) as the ratio of sporangia able to start a new disease cycle on grapevine tissues, the average value of IE% for the four experimental repetitions is 8.3 %.

In general, no sporulated leaf disks were scored before day 5, and the peak of infection was reached between day 7 (experimental repetition n.1) and 8 (experimental repetitions n. 2, 3 and 4) (Table 1), after which no new sporulated leaf disks were detected. The proportions of infected grapevine leaf discs calculated on the total for each population are close together and ranged from 6.25% in experimental repetition 1 and 2 to 10.4% of the remaining ones.

Table 1: cumulative daily number of sporangia able to infect grapevine leaf disks and infection efficiency (IE %) defined as the ratio of infected leaf disks over the total, for each of the 24-microtiter plate of the four experimental repetitions

Experimental repetition and plate		Days After Inoculation											IE %
		4	5	6	7	8	9	10	11	12	13	14	
Repetition 1	Plate 1.1	0	0	0	1	1	1	1	1	1	1	1	4.2
	Plate 1.2	0	0	1	1	1	1	1	1	1	1	1	4.2
	Plate 1.3	0	0	1	2	2	2	2	2	2	2	2	8.3
	Plate 1.4	0	0	2	2	2	2	2	2	2	2	2	8.3
Repetition 2	Plate 2.1	0	0	0	0	1	1	1	1	1	1	1	4.2
	Plate 2.2	0	0	0	1	1	1	1	1	1	1	1	4.2
	Plate 2.3	0	0	2	2	2	2	2	2	2	2	2	8.3
	Plate 2.4	0	0	1	2	2	2	2	2	2	2	2	8.3
Repetition 3	Plate 3.1	0	1	1	4	4	4	4	4	4	4	4	16.7
	Plate 3.2	0	1	1	3	4	4	4	4	4	4	4	16.7
	Plate 3.3	0	0	0	1	1	1	1	1	1	1	1	4.2
	Plate 3.4	0	0	1	1	1	1	1	1	1	1	1	4.2
Repetition 4	Plate 4.1	0	1	1	1	3	3	3	3	3	3	3	12.5
	Plate 4.2	0	0	0	0	0	0	0	0	0	0	0	0.0
	Plate 4.3	0	0	2	3	3	3	3	3	3	3	3	12.5
	Plate 4.4	0	0	1	4	4	4	4	4	4	4	4	16.7

The GLMM fitted above well describes the SID reproduced by the single-sporangia assay (pseudo-R^2=0.9911, Figure 7A).

All the simulated infection % values obtained at any DAI are included within the 95% confidence limits computed for the overall observed percentage of positively infecting sporangia, regardless the experiment. Overall, the Type II solution for the GLMM parameters (Table 2) shows a highly significant effect of time (DAI) on the response variable ($p(>\chi 2) < 0.001$), whereas the experiment's effect does not appear to significantly affect the SID ($p(>\chi 2) < 0.598$). On the other hand, glancing at Figure 7B, one can observe that t_{50} bootstrap empirical distribution approaches the normal density distribution. The bootstrap t_{50} computed value is 9.795 DAI, its 95% Lower Confidence Limit (LCL) is 9.632 DAI, and its Upper Confidence Limit (UCL) is 9.977 DAI. The GLMM t_{50} value is 9.797, thus it is included within the bootstrap t_{50} 95% confidence limit. Noteworthy, the difference between GLMM t_{50} and bootstrap t_{50} is 0.002 DAI, that is, 2 minutes and 53 seconds circa.

Figure 7: Sporangia germination trend in time for each experiment (A); comparison between bootstrap t_{50} and GLMM t_{50} (B).

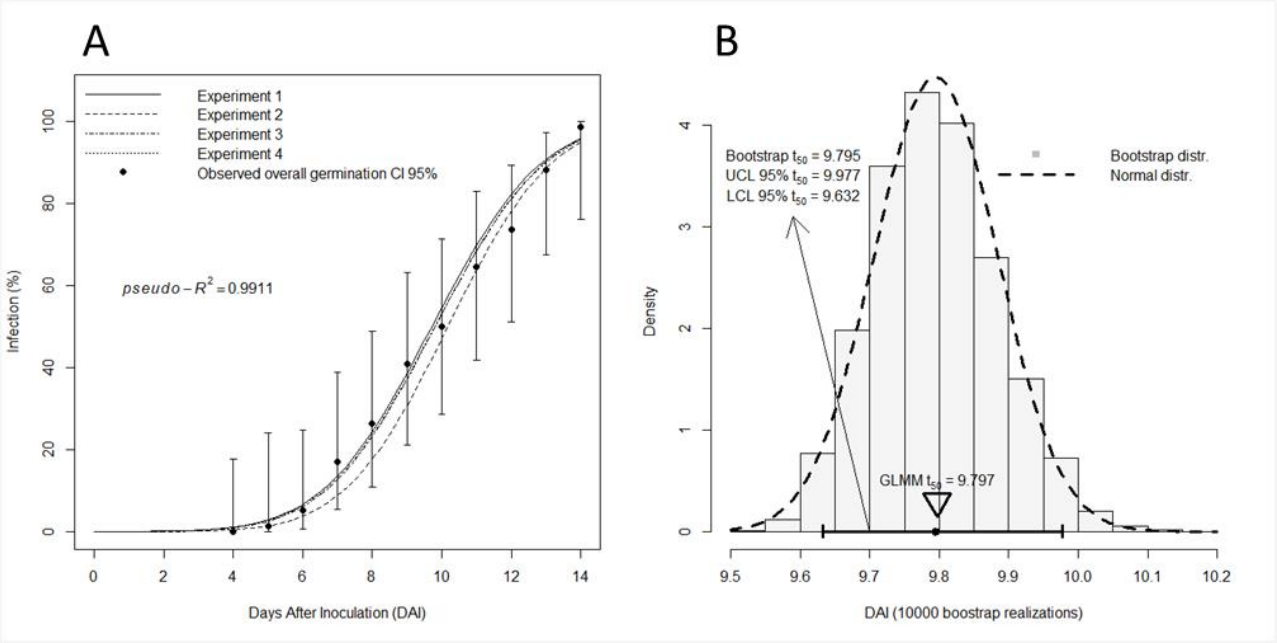


Table 2: Wald's χ^2 test (Type II solution) for GLMM fixed parameters.

Parameter	**χ^2**	**DF**	**$p(>\chi^2)$**
β_{ti} (time effect in Days After Inoculation)	2778.6480	1	<0.001
β_j (Experiment, with j={Experiment1, Experiment2,Experiment3, Experiment4}	1.8803	3	0.598

4. Discussion and conclusions

FCM technologies, and in particular FACS, have proven to be excellent tools that can be used in the study of oomycete sporangia suspensions. Many FCM applications, already developed in experiments performed on *P. infestans* by Day and coworkers (Day et al., 2002), such as recognition and counting of single sporangia within a suspension of heterogeneous particles, have also been successfully translated to *P. viticola*. However, the intrinsic red autofluorescence of *P.* viticola sporangia here reported did not allow the use of viability dyes, such as PI, to distinguish between spores with significant membrane damage and intact cells.

By combining the data obtained from FCM with direct observations under microscope, it was possible to find out that the composition of a suspension of sporangia obtained from grapevine leaves is not homogeneous and constantly in changing. An interesting detail in this regard is that the four subpopulations of particles within the suspension are connected together in a dynamic way. Indeed, testing the sporangia suspension 4 hours after the first experimental session, there was a shifting of particles among subpopulations probably linked to the biological features of the pathogen. The sporangia suspended in aqueous solutions at room temperature naturally tend to open and release the zoospores contained within them, so that they can move in the fluid through the movement of flagella to generate new infections (Gessler et al., 2011). This phenomenon was detected during our experiments by the changes in percentages between subpopulations. In this regard, it should be noted that despite double separate filtering of the PBS and of the sporangia suspension, it was not possible to prevent the presence of the debris and others foreign particles of no relevant interest composing the subpopulation III. This is a common problem also reported in other studies on oomycetes in which spores suspensions are obtained by washing leaf material (Day et al., 2002), and that we fixed by gating the data with polygonal regions drawn from plots of FSC versus SSC.

The use of FACS has proved to be practical and useful in evaluating the infection efficiency of the single sporangia composing the suspension. The developed tool has a precision much higher than traditional methods of isolation of single sporangia, which are based on manual techniques where an individual sporangiophore is picked up manually with a sterile pincer under microscope and then serially diluted in drops placed on untreated grapevine leaves (Massi et al., 2021; Toffolatti et al., 2018). In addition, considering that the traditional methodologies of isolations described above are highly time-consuming, the method here proposed can be used on large-scale populations investigations that would otherwise too much expansive in terms of resources to perform. On the other hand, the average yield of infecting sporangia here obtained (8.34%) is quite low, and lacking quantitative studies on sporangia infection process, with which to make a comparison, we cannot assert that the value here obtained is representative or not. Other investigations monitoring multiple populations are needed to contextualize these data, but it is not excluded that it can be increased. It is known indeed that sporangia productivity and germination of all leaf lesions decline rapidly through repeated cycles of sporulation and propagation (Kennelly et al., 2007). An increase in the infection yield could therefore be obtained by using fresh field suspensions (not propagated) which possess a greater infection capacity (Kennelly et al., 2004).

The GLMM fitted here is a good model to describe the experimental variable studied (the percentage of positively infected leaf disc) and its dynamics (SID) reproduced by single-sporangia assay, thus t_{50} is an appropriate index to summarize the median sporangia infection time. Given this assumption, the single experiment does not affect the Infection yield (as well the SID), and considering that the difference between GLMM t_{50} and bootstrap t_{50} is very short, the GLMM t_{50} is a good estimator for expected t_{50} arising from each of the plates randomly included in each experiment.

Overall, it can be concluded that single-sporangia assay as performed here is a precise and accurate quantitative experimental technique for sporangia isolation and infection efficiency evaluation, which allows to study the secondary infection cycles of the pathogen with greater completeness as it is currently possible to do for the primary ones. This represents a big step forward in the study of grapevine downy mildew since, to the best of our knowledge, all the experimental methodologies available to investigate sporangia infections do not provide quantitative data. The only exception to this statement is represented by few methods developed for quantification of sporangia germination, which are based on visual observation under microscope of zoospores release (Andrieu et al., 2001; Blum et al., 2010; Genet, 1999; Kennelly et al., 2007; Massi et al., 2021). However, the reliability of this type of data is quite limited, as it only evaluates the release and mobility of the zoospores in aqueous suspension without taking into account the outcome of the infection process on grapevines tissues. This information gap can be bridged using the method here proposed, which could be used in different fields of study of the pathogen such as predictive infection models or the quantification of the number of resistant strains in the context of fungicides resistance management.

5. Bibliography

Andrieu, N., Jaworska, G., Genet, J. L., & Bompeix, G. (2001). Biological mode of action of famoxadone on *Plasmopara viticola* and *Phytophthora infestans*. *Crop Protection*, *20*(3), 253–260. https://doi.org/10.1016/S0261-2194(00)00156-3

Blum, M., Boehler, M., Randall, E., Young, V., Csukai, M., Kraus, S., Moulin, F., Scalliet, G., Avrova, A. O., Whisson, S. C., & Fonne-Pfister, R. (2010). Mandipropamid targets the cellulose synthase-like PiCesA3 to inhibit cell wall biosynthesis in the oomycete plant pathogen, *Phytophthora infestans*. *Molecular Plant Pathology*, *11*(2), 227–243. https://doi.org/10.1111/j.1364-3703.2009.00604.x

Catal, M., King, L., Tumbalam, P., Wiriyajitsomboon, P., Kirk, W. W., & Adams, G. C. (2010). Heterokaryotic nuclear conditions and a heterogeneous nuclear population are observed by flow cytometry in *Phytophthora infestans*. *Cytometry Part A*, *77A*(8), 769–775. https://doi.org/https://doi.org/10.1002/cyto.a.20888

Corio-Costet, M.-F. (2015). Monitoring resistance in obligate pathogens by bioassays relating to field use: grapevine powdery and downy mildews. In *Fungicide Resistance in Plant Pathogens*. Springer Japan. https://doi.org/10.1007/978-4-431-55642-8_16

Czechowska, K., Johnson, D. R., & van der Meer, J. R. (2008). Use of flow cytometric methods for single-cell analysis in environmental microbiology. *Current Opinion in Microbiology*, *11*(3), 205–212. https://doi.org/https://doi.org/10.1016/j.mib.2008.04.006

D'hondt, L., Höfte, M., Van Bockstaele, E., & Leus, L. (2011). Applications of flow cytometry in plant pathology for genome size determination, detection and physiological status. *Molecular Plant Pathology*, *12*(8), 815–828. https://doi.org/https://doi.org/10.1111/j.1364-3703.2011.00711.x

Day, J. P., Kell, D. B., & Griffith, G. W. (2002). Differentiation of *Phytophthora infestans* sporangia from other airborne biological particles by flow cytometry. *Applied and Environmental Microbiology*, *68*(1), 37–45. https://doi.org/10.1128/AEM.68.1.37-45.2002

Díaz, M., Herrero, M., García, L. A., & Quirós, C. (2010). Application of flow cytometry to industrial microbial bioprocesses. *Biochemical Engineering Journal*, *48*(3), 385–407. https://doi.org/https://doi.org/10.1016/j.bej.2009.07.013

Dorai-Raj, S. (2014). *Binomial confidence intervals for several parameterizations, R package version 1.0-6*.

Efron, B., Farrell, T. R. J., Johnston, J. M., & Twynam, D. (1993). An introduction to the bootstrap. Chapman and Hall, New York, NY. *Farrell, J., Johnston, M. and Twynam, D.(1998),'"Volunteer Motivation, Satisfaction, and Management at an Elite Sporting Competition"', Journal of Sport Management*, *12*, 288–300.

Faraway, J. J. (2016). *Extending the linear model with R: generalized linear, mixed effects and*

nonparametric regression models. CRC press.

Fox, J. (2015). *Applied regression analysis and generalized linear models*. Sage Publications.

Genet, J L, Vincent, O. (1999). Sensitivity to Famoxate [famoxadone] of European *Plasmopara viticola* populations. *Agris*, *64*(3b), 559–564.

Gessler, C., Pertot, I., & Perazzolli, M. (2011). *Plasmopara viticola*: A review of knowledge on downy mildew of grapevine and effective disease management. *Phytopathologia Mediterranea*, *50*(1), 3–44. https://doi.org/10.14601/Phytopathol_Mediterr-9360

Guyot, S., Gervais, P., Young, M., Winckler, P., Dumont, J., & Davey, H. M. (2015). Surviving the heat: heterogeneity of response in *Saccharomyces cerevisiae* provides insight into thermal damage to the membrane. *Environmental Microbiology*, *17*(8), 2982–2992. https://doi.org/https://doi.org/10.1111/1462-2920.12866

Hill, G. K. (1998). Studies on the germination of *Plasmopara viticola* oospores with a floating disc test. *Bulletin OILB I SRO*, *21*, 1–2.

Ishii, S., Tago, K., & Senoo, K. (2010). Single-cell analysis and isolation for microbiology and biotechnology: methods and applications. *Applied Microbiology and Biotechnology*, *86*(5), 1281–1292. https://doi.org/10.1007/s00253-010-2524-4

Kennelly, M. M., Seem, R. C., Gadoury, D. M., Wilcox, W. F., & Magarey, P. A. (2004). Survival of grape downy mildew (*Plasmopara viticola*) sporangia and lesions under field conditions. *Phytopathology*, *94*(S50).

Kennelly, Megan M., Gadoury, D. M., Wilcox, W. F., Magarey, P. A., & Seem, R. C. (2007). Primary infection, lesion productivity, and survival of sporangia in the grapevine downy mildew pathogen *Plasmopara viticola*. *Phytopathology*, *97*(4), 512–522. https://doi.org/10.1094/PHYTO-97-4-0512

Lafon, R., & Clerjeau, M. (1988). Downy mildew. In R. C. Pearson & A. C. Goheen (Eds.), *Compendium of grape diseases* (pp. 11–13). APS Press.

Lehoczky, J. (1965). Observations on oospore production by *Plasmopara viticola* in floating leaf discs in artificial culture. *Vitis*, *5*, 17–19.

Maddalena, G., Delmotte, F., Bianco, P. A., De Lorenzis, G., & Toffolatti, S. L. (2020). Genetic structure of Italian population of the grapevine downy mildew agent, Plasmopara viticola. *Annals of Applied Biology*, *176*(3), 257–267. https://doi.org/https://doi.org/10.1111/aab.12567

Maddalena, G., Russo, G., & Toffolatti, S. L. (2021). The Study of the Germination Dynamics of *Plasmopara viticola* Oospores Highlights the Presence of Phenotypic Synchrony With the Host. *Frontiers in Microbiology*, *12*, 698586. https://doi.org/10.3389/fmicb.2021.698586

Massi, F., Torriani, S. F. F., Borghi, L., & Toffolatti, S. L. (2021). Fungicide resistance evolution and detection in plant pathogens: *Plasmopara viticola* as a case study. *Microorganisms*, *9*(1), 1–

18. https://doi.org/10.3390/microorganisms9010119

Piñeiro, G., Perelman, S., Guerschman, J. P., & Paruelo, J. M. (2008). How to evaluate models: Observed vs. predicted or predicted vs. observed? *Ecological Modelling*, *216*(3), 316–322. https://doi.org/https://doi.org/10.1016/j.ecolmodel.2008.05.006

Rossi, V., & Caffi, T. (2007). Effect of water on germination of *Plasmopara viticola* oospores. *Plant Pathology*, *56*(6), 957–966. https://doi.org/https://doi.org/10.1111/j.1365-3059.2007.01685.x

Rüger, M., Bensch, G., Tüngler, R., & Reichl, U. (2012). A flow cytometric method for viability assessment of *Staphylococcus aureus* and *Burkholderia cepacia* in mixed culture. *Cytometry Part A*, *81A*(12), 1055–1066. https://doi.org/https://doi.org/10.1002/cyto.a.22219

Sargolzaei, M., Maddalena, G., Bitsadze, N., Maghradze, D., Bianco, P. A., Failla, O., Toffolatti, S. L., & De Lorenzis, G. (2020). *Rpv29, Rpv30* and *Rpv31*: Three novel genomic loci associated with resistance to *Plasmopara viticola* in *Vitis vinifera. Frontiers in Plant Science*, *11*. https://doi.org/10.3389/fpls.2020.562432

Sergeeva, V., Nair, N., Legendre, L., Darley, E., & Spooner-Hart, R. (2002). The use of fluorochromes to determine the effect of chlorine dioxide on survival of *Plasmopara viticola* on grapevine. *Australasian Plant Pathology*, *31*(3), 295–297. https://doi.org/10.1071/AP02018

Shapiro, H. M. (2005). *Practical flow cytometry*. John Wiley & Sons.

Si Ammour, M., Bove, F., Toffolatti, S. L., & Rossi, V. (2020). A Real-Time PCR Assay for the quantification of *Plasmopara viticola* oospores in grapevine Leaves. *Frontiers in Plant Science*, *11*, 1202. https://doi.org/10.3389/fpls.2020.01202

Toffolatti, S. L., Serrati, L., Sierotzki, H., Gisi, U., & Vercesi, A. (2007). Assessment of QoI resistance in *Plasmopara viticola* oospores. *Pest Management Science*, *63*(2), 194–201. https://doi.org/https://doi.org/10.1002/ps.1327

Toffolatti, S. L., De Lorenzis, G., Costa, A., Maddalena, G., Passera, A., Bonza, M. C., Pindo, M., Stefani, E., Cestaro, A., Casati, P., Failla, O., Bianco, P. A., Maghradze, D., & Quaglino, F. (2018). Unique resistance traits against downy mildew from the center of origin of grapevine (*Vitis vinifera*). *Scientific Reports*, *8*(1), 12523. https://doi.org/10.1038/s41598-018-30413-w

Toffolatti, S. L., Prandato, M., Serrati, L., Sierotzki, H., Gisi, U., & Vercesi, A. (2011). Evolution of QoI resistance in Plasmopara viticola oospores. *European Journal of Plant Pathology*, *129*(2), 331–338. https://doi.org/10.1007/s10658-010-9677-y

Toffolatti, S. L., Russo, G., Campia, P., Bianco, P. A., Borsa, P., Coatti, M., Torriani, S. F., & Sierotzki, H. (2018). A time-course investigation of resistance to the carboxylic acid amide mandipropamid in field populations of *Plasmopara viticola* treated with anti-resistance strategies. *Pest Management Science*, *74*(12), 2822—2834. https://doi.org/10.1002/ps.5072

Vercesi, A., Toffolatti, S. L., Zocchi, G., Guglielmann, R., & Ironi, L. (2010). A new approach to

modelling the dynamics of oospore germination in *Plasmopara viticola*. *European Journal of Plant Pathology*, *128*(1), 113–126. https://doi.org/10.1007/s10658-010-9635-8

Vercesi, A., Tornaghi, R., Sant, S., Burrano, S., & Faoro, F. (1999). A cytological and ultrastructural study on the maturation and germination of oospores of *Plasmopara viticola* from overwintering vine leaves. *Mycological Research*, *103*(2), 193–202. https://doi.org/10.1017/S095375629800700X

Vercesi, A., Vavassori, A., Faoro, F., & Bisiach, M. (2002). Effect of azoxystrobin on the oospores of *Plasmopara Viticola.* In P. T. N. Spencer-Phillips, U. Gisi, & A. Lebeda (Eds.), *Advances in Downy Mildew Research* (pp. 195–199). Springer Netherlands. https://doi.org/10.1007/0-306-47914-1_11

Winson, M. K., & Davey, H. M. (2000). Flow Cytometric Analysis of microorganisms. *Methods*, *21*(3), 231–240. https://doi.org/https://doi.org/10.1006/meth.2000.1003