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ORIGINAL ARTICLE

The management of grapevine downy mildew: from anti-resistance strategies to innovative approaches for fungicide resistance monitoring

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Abstract

Efficient grapevine downy mildew control necessitates the implementation of anti-resistance strategies to ensure the ongoing efficacy of available substances and optimal disease control. With the gradual disappearance of multi-site fungicides from the market, reliance on single-site fungicides poses a long-term risk of selecting strains resistant to multiple modes of action. Challenges in disease management encompass selecting optimal spray programs and monitoring field population sensitivity. This study evaluated the efficacy of anti-resistance strategies, including two single-site fungicides (mandipropamid and oxathiapiprolin), on disease control and fungicide sensitivity through a combination of field trials and laboratory tests for the biological and molecular characterization of the pathogen populations over a three-year period (2019–2021). Mandipropamid, a cellulose synthase inhibitor, is used since a long time for downy mildew control, while oxathiapiprolin, an OxySterol Binding Protein homologue Inhibitor, was introduced recently. Field trials demonstrated effective disease control, even in the presence of mandipropamid-resistant strains (with G1105S/V mutations in *PvCesA3*) and revealed a pronounced selection and spread of resistance to both fungicides in the vineyard where disease pressure was higher. Characterizing pathogen strains remained a significant obstacle in sensitivity monitoring, hindering precise determination of resistance frequencies related to fungicide programs. Traditional techniques, in fact, lack the resolution required for high-throughput isolation and characterization of resistant individuals. To address this challenge, we propose utilizing flow cytometry and fluorescence-activated cell sorting on field sporangia populations, a method able to determine both the number of resistant isolates and isolate pathogen strains in a single assay.

Keywords Plant disease · Oomycete · Disease control · Integrated pest management

Introduction

Downy mildew, caused by the oomycete *Plasmopara viticola* (Berk. *et* Curtis) Berl. and De Toni, is considered one of the most serious diseases in viticulture worldwide (Bois et al. 2017). Given the high susceptibility of grapevines (*Vitis vinifera* L.) to *P. viticola*, disease control is of paramount importance to the wine industry (Gessler et al. 2011). Research efforts have resulted in the integration of agronomic, genetic, and chemical means and primary elements for successfully reducing the damage caused by this pathogen (Vezzulli et al. 2018; Bregaglio et al. 2022; Eisenmann et al. 2023; Maddalena et al. 2023).

Despite the efforts of geneticists to obtain resistant cultivars and the adoption of cultural practices aimed at reducing

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conditions favoring pathogen development, chemical control based on the use of organic and inorganic fungicides currently represents the most effective and widespread measure for protecting grapevines. Many of the active ingredients used for downy mildew control belong to fungicide groups with a single-site mode of action, the use of which is associated with a high risk of selecting resistant strains within the pathogen population. Indeed, resistance to most fungicide classes has been reported (Massi et al. 2021; Mboup et al. 2021; Cherrad et al. 2023). To preserve the effectiveness of the active substances on the market, it is important and necessary to investigate the mechanisms that govern the emergence of such resistances and to monitor the spread of resistant strains. Fungicide resistance is, in fact, an evolutionary phenomenon affecting organisms subjected to selection pressure from one or more fungicide classes (Hollomon and Brent 2009; Hobbelen et al. 2014). The risk of resistance emergence results from a combination of various factors (Hollomon 2015), including the biology of the pathogen (monocyclic vs. polycyclic), the mode of action of the fungicide (single-site vs. multi-site), and its frequency of use. Resistance is promoted by the repeated use of the same chemical product in the field, while its origin varies but is mostly attributable to changes in the genetic composition of the pathogen population (Delmas et al. 2017; Pereira et al. 2021; Yin et al. 2023).

The implementation of anti-resistance strategies (Brent and Hollomon 2007) is crucial for efficiently controlling grapevine downy mildew, and this is due to several reasons. Firstly, *P. viticola* is a high-risk, polycyclic pathogen (FRAC - Fungicide Resistance Action Committee 2019). Secondly, the most economically relevant cultivars are highly susceptible to the pathogen, and disease management mostly relies on chemical control. Lastly, multi-site (low-risk) fungicides are gradually disappearing from the market, shifting the control of the disease to single-site fungicides. The reduction in the variety of fungicide modes of action increases the difficulty in managing resistance for the remaining ones (Corkley et al. 2022). Therefore, from a practical point of view, preserving the efficacy of a class is crucial to maintain the effectiveness of the other ones, particularly when anti-resistance strategies mostly rely in mixture/alternation of single-site fungicides. In the long-term, the selection of strains resistant to multiple modes of action must also be considered (Santos et al. 2020). Consequently, the challenges in downy mildew management range from selecting the best spray program to monitoring the sensitivity of field populations, taking into considerations that strategies to manage the evolution of resistance must not only reduce the resistant subpopulation, but also overall disease levels (Hollomon 2015).

This study focused on two fungicides, mandipropamid and oxathiapiprolin, which possess different modes of action (FRAC MoA working group 2022) and were subjected to different levels of selection pressure through time. The cellulose synthase inhibitor mandipropamid belongs to the CAA (carboxylic acid amides) group, which has been used for downy mildew control for decades. In contrast, oxathiapiprolin belongs to the OSBPI (OxySterol Binding Protein homologue Inhibitors) group, which was introduced into the market at the start of this study. As a result, *P. viticola* field populations have been exposed to long-term selection pressure from the CAA group, while no selection pressure was previously applied by the OSBPI group. The aims of our study were: (i) to evaluate the effectiveness of an anti-resistance strategy involving mandipropamid to control *P. viticola* infections in the field; (ii) to investigate the impact on the sensitivity levels of the population of the pathogen; and (iii) to establish the sensitivity of the pathogen to oxathiapiprolin.

Materials and methods

Field trials

The activities were conducted during the grapevine growing seasons from 2019 to 2021 in two commercial vineyards: one located in Friuli, at Zoppola (PN), and the other in Lombardy, at Santa Maria della Versa (PV). Two anti-resistance strategies, differing only in the application of CAA mandipropamid, were implemented in two plots, each covering 1–2 hectares (Supplementary Table 1). Strategy A included three treatments with mandipropamid in a mixture and alternated with other modes of action. Strategy B did not involve any CAA treatments. Both strategies included two treatments with oxathiapiprolin. The treatments were carried out by the farmers using their own equipment. In addition, an untreated plot, consisting of five rows, 20 m long in Zoppola, and three rows, 50 m long in Santa Maria della Versa, was established. Disease incidence and severity were assessed in the three plots at bunch closure (Toffolatti et al. 2018). Furthermore, environmental conditions, including temperature and rainfall, were monitored through weather stations placed within each vineyard.

Sensitivity assays

Characterization of the populations involved determining the EC_{50} (median effective concentration) values for mandipropamid and oxathiapiprolin and quantifying the point mutations associated with resistance to mandipropamid in the *PvCesA3* gene. The tests were conducted on 100 infected leaves sampled within each plot at bunch closure.

Biological assays were carried out following procedures described in previous works (Toffolatti et al. 2018; Massi et al. 2023). These assays involved the inoculation

of sporangia on leaf disks treated with mandipropamid (at concentrations of 0, 0.01, 0.1, 1, 10, and 100 mg/L) or oxathiapiprolin (at concentrations of 0, 0.00004, 0.0004, 0.004, 0.04, and 0.4 mg/L). EC_{50} values were estimated through dose–response analysis of disease severity (Toffolatti et al. 2018). EC_{50} values exceeding 10 mg/L were indicative of resistance to mandipropamid (Sierotzki et al. 2011).

P. viticola strains were obtained through serial dilution of sporangia derived from individual sporangiophores collected from the 2019 samples (Massi et al. 2022). The strains were propagated weekly until enough sporangia material for molecular assays was obtained.

The percentage of allelic variants at codon 1105 of the *PvCesA3* gene, associated with sensitivity (G1105) or resistance (S1105 and V1105) to mandipropamid, was assessed in *P. viticola* populations and strains. Real-time PCR was performed on DNA extracted from sporangia (Toffolatti et al. 2018) using the primers listed in Table 1. The phenotypes of the strains were predicted based on allelic composition (Sierotzki et al. 2011). Sensitive strains were characterized by the G1105 allele in homozygosis (GG genotype) or in heterozygosis with S1105 (GS genotype) or V1105 (GV genotype). Resistant strains showed the presence of S1105 or V1105 alleles in homozygosis (SS or VV genotypes) or in heterozygosis (SV genotype). Due to the low amount of DNA, samples from strategy A plot could not be characterized molecularly in 2019 (Zoppola vineyard) and 2021 (Santa Maria della Versa vineyard).

Data analysis

Significant differences between disease incidence and severity values of the treated plots were assessed through one-way ANOVA by using SPSS v. 28 (IBM Statistics, Milano, Italy). Significant differences among allele percentages of the samples belonging to different plots were assessed through Kruskal–Wallis test ($P = 0.05$) by using SPSS v. 28.

Table 1 List of the forward (FW) and reverse (REV) primers used to amplify allelic variants (G, S, and V) at codon 1105 of the *PvCesA3* gene

Primer	Sequence (5'-3')	Length
G_FW	CCT TTA CGG CAA ATG TGT TAG G	22
S_FW	ACC TTT ACG GCA AAT GTG TTG A	22
V_FW	ACC TTT ACG GCA AAT GTG TTC TT	23
REV	CCA ACA AGT TGC CCT CGT AAT	21

Results

Field trials

As shown in Table 2, the environmental conditions in the first vineyard (Zoppola) were highly conducive to disease onset, with mild temperatures and a significant amount of rainfall occurring from April to July. Consequently, disease severity and incidence values in the untreated plot exceeded 75% and 95%, respectively (Table 3). Conversely, the reduced amount of rainfall in Santa Maria della Versa (Table 2) limited disease intensity, with severity remaining below 9.5% and incidence below 50% (Table 3). In the treated plots of both vineyards, disease severity was below 2.8% and 1% on leaves and bunches, respectively, while disease incidence ranged from 0.1 to 11% on leaves and from 0.1 to 3% on bunches. No significant differences were found between treated plots within and between vineyards for both disease severity and incidence on leaves and bunches ($P > 0.05$).

Sensitivity assays

The EC_{50} values of *P. viticola* populations sampled in Zoppola exceeded 100 mg/L for mandipropamid, indicating the presence of resistant strains. Molecular analyses showed a high frequency of S1105 and V1105 alleles within the population (Fig. 1a) and a high frequency of resistant individuals, particularly with the SV phenotype (Table 4). No significant differences were found among plots for the percentages of G/S/V1105 alleles ($P > 0.41$). In contrast, no resistance to

Table 2 Monthly average values of temperature (°C) and sum of rain (mm) recorded from April to July in 2019–2021 in the two vineyards

		Zoppola (PN)		Santa Maria della Versa (PV)	
Year	Month	Temperature (C°)	Rain (mm)	Temperature (C°)	Rain (mm)
2019	April	13.1	268.4	11.7	88
	May	14.7	234.4	14.0	197.8
	June	24.2	9.8	22.7	9.4
	July	23.5	112.4	24.5	11.8
2020	April	14.5	37.6	12.0	46.2
	May	17.2	102	17.7	38.6
	June	20	239.4	20.3	67.2
	July	22.8	108.4	22.6	40
2021	April	11	71.6	10	58.2
	May	15	270.2	15.2	39.2
	June	22.9	59	22.2	11.6
	July	23.2	28.6	22	5.2

Table 3 Disease severity (%) and incidence (%) on leaves and bunches of the three plots of the two vineyards from 2019 to 2021. The EC_{50} values (mg/L) of the populations sampled in each plot, both for mandipropamid and oxathiapiprolin, are also reported

Vineyard	Year	Plot	Severity (%)		Incidence (%)		EC_{50} (mg/L)	
			Leaves	Bunches	Leaves	Bunches	Oxathiapiprolin	Mandipropamid
Zoppola	2019	UNTREATED	81	83.3	96.5	100	0.00002	> 100
		STRATEGY A	0.3	0	1.3	0.1	0.02	> 100
		STRATEGY B	2.3	0.1	7	1.3	0.01	> 100
	2020	UNTREATED	89.1	96.9	99	100	0.07	> 100
		STRATEGY A	2.3	1	5	2	> 4	> 100
		STRATEGY B	0	0.1	0.1	0.5	0.006	> 100
	2021	UNTREATED	75	93.5	97.5	99.5	0.25	> 100
		STRATEGY A	1	0	3	0.1	> 0.4	> 100
		STRATEGY B	0.3	0.1	1	0.5	> 0.4	> 100
Santa Maria della Versa	2019	UNTREATED	8.2	0.4	21.7	0.7	0.01	7.1
		STRATEGY A	2.8	0.2	9	0.7	0.01	15.5
		STRATEGY B	1.8	0.3	11	3	0.02	0.02
	2020	UNTREATED	9.5	1.2	50	7	0.01	0.01
		STRATEGY A	0.42	0.14	1	0.5	0.06	0.06
		STRATEGY B	0.67	0.14	1.5	0.5	0.01	0.01
	2021	UNTREATED	1	0.2	1.5	0.75	< 0.01	< 0.01
		STRATEGY A	0.35	0	0.35	0.1	< 0.01	< 0.01
		STRATEGY B	0.9	0.35	0.9	0.25	< 0.01	0.03

mandipropamid was detected in Santa Maria della Versa, except for the Strategy A plot in 2019 (Table 3). In this case, the EC_{50} value was 15.5 mg/L, just slightly above the 10 mg/L threshold, and a significantly lower ($P = 0.042$) frequency of G1105 allele was detected compared to the samples of the other two plots (Fig. 1b). Furthermore, SS, SV, and VV individuals were present within the population (Table 4). The remaining populations were generally characterized by a 50% presence of G1105 allele and no significant differences among plots for the allelic composition ($P > 0.09$) (Fig. 1b).

The EC_{50} values for oxathiapiprolin were generally below 0.06 mg/L (Table 3). Importantly, in 2020 and 2021, the EC_{50} values for the active ingredient were more than 20 times greater than those observed in 2019, which was the first year of oxathiapiprolin application, in the Zoppola vineyard.

Discussion

The results of this work provide significant insights into the influence of environmental conditions and disease intensity on the fungicide sensitivity of *P. viticola* populations. In the Zoppola vineyard, where weather conditions led to high disease pressure, the pathogen population consistently maintained high resistance to mandipropamid over time, as demonstrated by the elevated EC_{50} values, and the frequent occurrence of S/V1105 alleles and resistant genotypes. Similar allelic compositions were observed in plots treated with mandipropamid (Strategy A plot) and untreated plots (Untreated and Strategy B plots). The persistence of resistance in untreated plots could be attributed to the long-term use of the CAA class in the vineyard, which may have led to the selection of resistant strains before the start of this project.

In Santa Maria della Versa, the pathogen population in the mandipropamid-treated plot (Strategy A) initially showed EC_{50} values indicative of resistance and a significantly different allelic composition compared to the other two plots. However, under conditions of particularly low disease pressure in 2020 and 2021, the field population in the Strategy A plot reverted to sensitivity. This was evidenced by EC_{50} values falling below the 10 mg/L threshold and a G1105 allele frequency analogous to that of plots not treated with mandipropamid. The change over time in the population composition could be the result of the reduction of the pathogen infection rate caused by the absence of rainy events necessary for the pathogen to colonize grapevine tissues (Fröbel and Zyprian 2019). Modeling studies indeed demonstrated that a reduction in the growth rate of both sensitive and resistant strains, or resistant strains alone, minimizes the selection of resistant strains (Van Den Bosch et al. 2014; Corkley et al. 2022). The increase in G1105 allele in the Strategy A plot suggests that the reduced disease pressure in Santa Maria

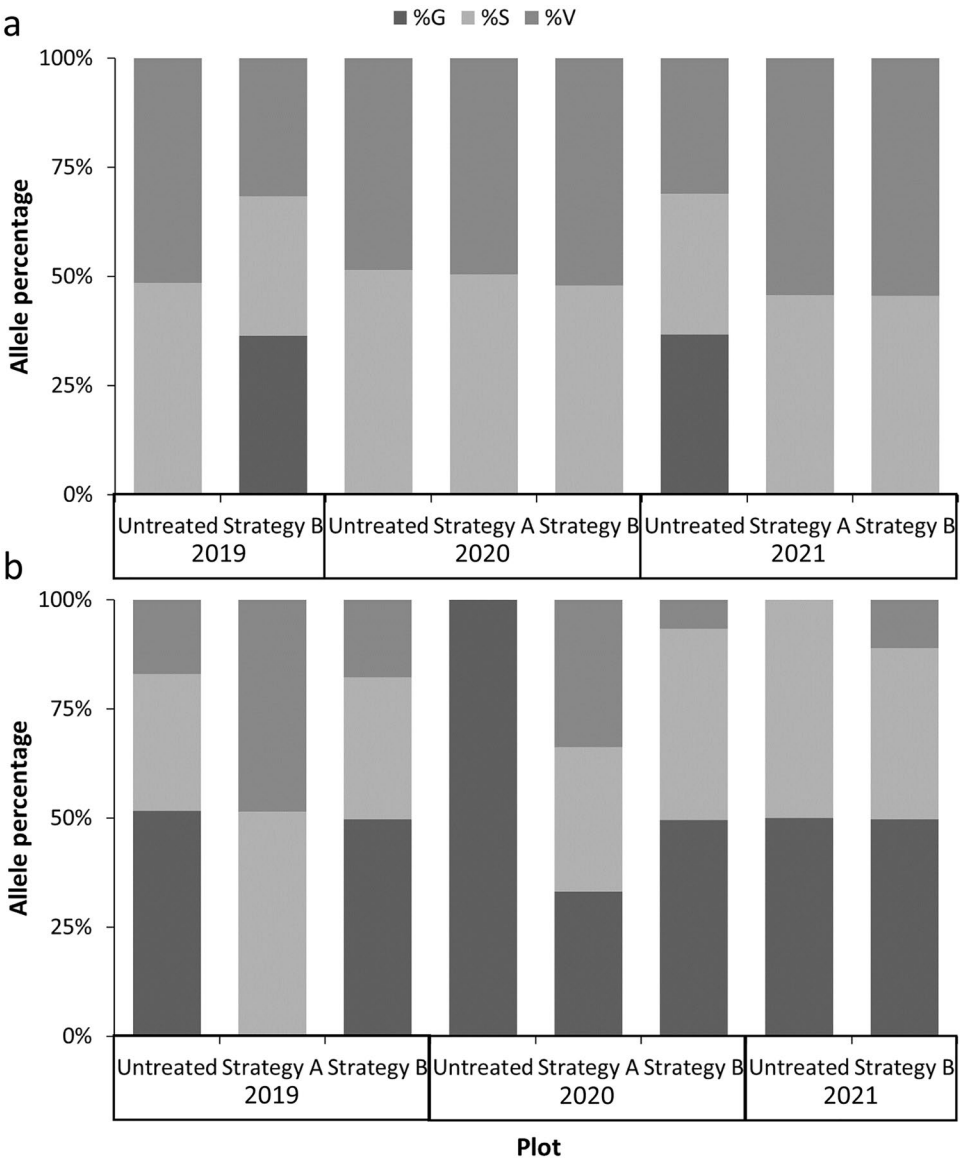

Fig. 1 Percentage of G1105 (%G), S1105 (%S), and V1105 (%V) alleles within *P. viticola* populations sampled from the different plots of Zoppola (**a**) and Santa Maria della Versa (**b**) vineyards, 2019–2021

Table 4 Number of *P. viticola* strains with sensitive (GG, GS, and GV) and resistant (SS, SV, and VV) genotypes to mandipropamid among those isolated from the different plots of the two vineyards

Number of isolates	Vineyard	Plot	Genotype					
			GG	GS	GV	SS	SV	VV
5	Zoppola	Strategy A	0	0	0	2	3	0
20		Untreated	0	0	0	1	12	7
10	Santa Maria della Versa	Strategy A	0	0	0	4	5	1
14		Untreated	1	2	1	2	7	1

della Versa mostly associated with a reduced infection rate by resistant strains. Previous field trials indicated a decline in resistance in the absence of CAA treatments, suggesting that resistant individuals may exhibit lower fitness compared to sensitive individuals (Gisi and Sierotzki 2008). The lack of information on the genotype composition of *P. viticola* individuals within the populations and on the fitness of resistant strains, however, prevents a definitive conclusion on this matter.

Biological and molecular assays on populations are useful for detecting shifts in pathogen sensitivity, but do not provide insights on the genotypes that have been selected by the treatment strategy. One of the challenges encountered in characterizing resistance to fungicides in populations of

P. viticola is, in fact, the isolation of a sufficient number of pathogen strains that accurately represent the real field situation. The common protocol for strain isolation from single sporangiophores is not precise, since it is not possible to verify whether the achieved isolate truly derives from the germination of a single sporangium (Massi et al. 2022). Furthermore, the propagation of the strains is a time-consuming procedure, taking, on average, six weeks to obtain enough material for sensitivity assays. Additionally, the isolation procedure is not always successful, and strains can be lost over time during the weekly propagation. This invariably leads to a reduced number of selected strains that may not be representative of the real field population. In this study, for instance, we isolated more than 100 strains but could only characterize half of them from four out of six plots. Recently, a protocol based on flow cytometry and fluorescence-activated cell sorting has been developed for the high-throughput isolation and characterization of *P. viticola* strains (Massi et al. 2022). This method could be employed for fungicide sensitivity tests, where single sporangia are selected and inoculated on leaf disks treated with increase in concentrations of fungicide. This method enables the concurrent assessment of multiple factors (Fig. 2). It allows for the determination of EC_{50} values in sporangia populations by analyzing disease severity at various fungicide concentrations (dose–response analysis). Additionally, it facilitates the identification and isolation of fungicide-resistant strains through discriminatory-dose assays (*i.e.*, assessing pathogen growth at specific doses where only resistant strains can survive). This approach ensures a highly precise and throughput isolation of strains from individual sporangia that can be immediately used for molecular analyses and/or further propagated to evaluate fitness traits.

As compared with disease protection in a different vineyard (Toffolatti et al. 2018), no differences were noted in Zoppola and Santa Maria della Versa, despite variations in sensitivity to mandipropamid. It is crucial to highlight that this outcome is a direct result of the adoption of an anti-resistance strategy that incorporates the use of mixtures and alternations of fungicides with different modes of action. In this strategy, mandipropamid-resistant strains are eliminated by the fungicide partners, and reciprocally, mandipropamid eliminates strains resistant to the fungicide partners. A strategy relying solely on mandipropamid could have yielded different outcomes, potentially resulting in ineffective disease control. A similar situation in field trials, where resistance to QoI fungicides was confirmed by the prevalent G143A mutation in the cytb gene, demonstrated that using QoI fungicides did not provide adequate disease protection (Campbell et al. 2021).

The baseline sensitivity of oxathiapiprolin in the two vineyards ranged from 0.01 to 0.25 mg/L, aligning with the range previously reported for Italian vineyards (Mboup et al. 2021; Massi et al. 2023). However, the EC_{50} values

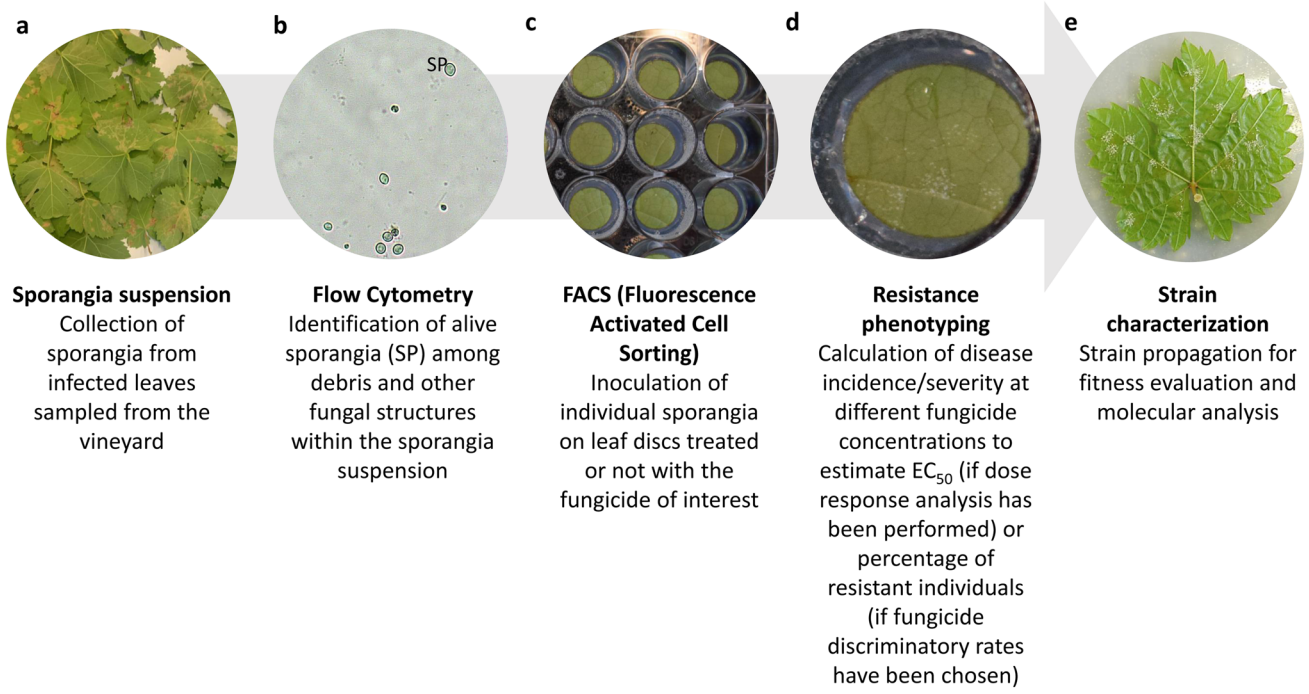

Fig. 2 Proposed workflow based on flow cytometry **b** and cell sorting **c** to characterize *P. viticola* populations **a** and simultaneously isolate individual strains **d** for characterization **e** in terms of biological (fungicide sensitivity and fitness) and molecular features (mutations associated with resistance)

exceeding 0.4 mg/L in Zoppola during the third year of oxathiapiprolin application suggested a shift toward less sensitive values after a few years of fungicide utilization. Notably, two strains from this vineyard exhibited the N752I mutation in *PvORP1* (homologous to the N837I mutation found in *Phytophthora infestans* (Mont.) De Bary) linked to resistance to the fungicide (Massi et al. 2023). The rapid emergence of resistant individuals aligns with previous observations for other fungicide classes, such as QoI, in *P. viticola* populations (Gisi et al. 2002; Grimmer et al. 2014). This finding underscores the need for a rigorous fungicide resistance management protocol. In this vineyard, the selection of resistant strains should be minimized by reducing the frequency of fungicide applications, avoiding two consecutive sprays, and diversifying the sprays with alternative modes of action.

In conclusion, this study demonstrates that although the selection of resistant strains cannot be completely avoided, the strict adoption of anti-resistance criteria results in an optimal disease protection. However, particular attention should be taken in those seasons and/or locations characterized by a high disease pressure, since in these conditions the selection and spread of resistant strains seems to be more pronounced. In this view, more emphasis should be given to alternative disease control measures such as disease forecasting models, which may help identifying the right moment for fungicide spray avoiding unnecessary treatments. High-throughput, quantitative information on the composition of pathogen populations at the biological and molecular levels will undoubtedly be valuable for evaluating the effects of disease management on the selection of resistant individuals. This information is important to develop precision strategies for resistance management by supporting the choice of appropriate modes of action and detecting outbreaks of strains resistant to single or multiple fungicides.

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Declarations

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