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

Running title: Oxathiapiprolin resistance in Italian vineyards

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Abstract

Background: 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 samples collected in ten different viticultural areas. Two insensitive strains were characterized for their mechanism of resistance.

Results: Oxathiapiprolin exhibited substantial inhibitory activity against 27 of the 29 populations tested, with EC_{50} values ranging from a minimum of under $4x10^{-5}$ mgL^{-1} to over $4x10^{-1}$ mgL^{-1}, with an average value of $3.2x10^{-2}$ mgL^{-1}. Two stable suspected oxathiapiprolin-resistant mutants were isolated from population exhibiting reduced sensitivity, and sequenced for the oxathiapiprolin target gene *PvORP1*. The comparison with wild-type isolates revealed that the resistant isolates possessed a heterozygous mutation causing the amino acid substitution N837I, recently reported in the literature.

Conclusion: The results obtained indicate a risk for Italian *P. viticola* populations to develop resistance to oxathiapiprolin in association with the N837I mutation at *PvORP1*. Anti-resistance strategies should be carefully implemented and the sensitivity levels to this molecule should be monitored accurately in future to preserve its effectiveness.

Keywords: fungicide resistance; grapevine; downy mildew; resistance monitoring; mechanism of fungicide resistance.

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.[1] *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,[2] the growing of traditional varieties is difficultly conceivable without frequent fungicide applications, since chemical control of the pathogen still represents the most important measure to ensure an adequate yield.[3]

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 currently available for chemical control of grapevine downy mildew.[4] The main mechanism of resistance is linked to single nucleotide polymorphisms (SNPs) in the gene encoding the target fungicide that cause a decrease in sensitivity.[5] In order to preserve the effectiveness of such compounds, fungicide resistance must be carefully managed, and for this purpose, the monitoring of *P. viticola* populations for their sensitivities to the different active principles plays a key role in resistance management.[6]

Oxathiapiprolin was the first of the piperidinyl-thiazole-isoxazoline fungicides to be discovered,[7] and has been shown to be highly effective against a large number of plant pathogenic oomycetes, including, *P. viticola*.[8–10] Binding assays and affinity chromatography carried out on oxathiapiprolin have shown that the intracellular target of this fungicide is one of the members of the oxysterol binding protein (OSBP)-related proteins (ORPs) family.[7,11,12] 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.[13] One of the main parameters 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. The availability of the baseline 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.[14]

To date there are few data available regarding the potential of *P. viticola* to evolve resistance to oxathiapiprolin and few reports of single nucleotide polymorphisms (SNPs) in the gene encoding for the fungicide target linked to a possible decrease in sensitivity.[7,9,15] In particular, Mboup et al. (2021)[15] 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 oxathiapiprolin; and (ii) investigate oxathiapiprolin-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 randomly collected from each vineyard at different times of the grapevine growing season (Table 1), depending on the availability of inoculum. At least 10 distant rows were sampled and 2-5 single leaves were collected per row. Information on the number of treatments carried out

with oxathiapiprolin 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 by farmers with commercial formulations at the doses indicated on the product labels and with their own equipment. Of the 29 *P. viticola* populations, 12 were collected from vineyards treated 1-3 times with oxathiapiprolin, two from vineyards where it was applied 4 times and 15 from fields where it had not been applied during the monitoring year. However, in one of these latter vineyards (Pv-26), oxathiapiprolin had been applied for five consecutive years prior to 2019.

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 collected by shaking the leaves one by one in a glass beaker containing 100 mL of sterile-distilled water: part of the suspension was immediately used for the sensitivity assay and the rest was centrifuge to remove water and retrieve the sporangia which were 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,[16] 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. Oxathiapiprolin, technical grade (96.7%, active ingredient [a.i.]), was 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

107 mgL^{-1} stock solution, which was stored in darkness at 4°C until serially dilution in double-distilled
108 sterile water (ddH_2O) to obtain the desired fungicide concentrations for sensitivity test. The
109 fungicide concentrations used for field populations were 0, $4x10^{-5}$, $4x10^{-4}$, $4x10^{-3}$, $4x10^{-2}$, $4x10^{-1}$ mgL^{-1}. The final concentration of DMSO was below 0.1% (v/v), a concentration that, according to
111 preliminary tests, does not cause any negative effect on the pathogen.[17,18]

112 After fungicide spraying, the treated leaf discs were dried in a flow hood and inoculated with the
113 sporangia suspensions by evenly spraying the suspension onto the leaf discs and incubating them in
114 a humid chamber at 20–22 °C with a 12:12 h photoperiod. Each leaf disc was scored for the area
115 affected by sporulation 9 days after inoculation, and disease severity (I%I),[3] was calculated for each
116 fungicide concentrations. Percentage inhibition of sporulation (IS) by oxathiapiprolin was calculated
117 with the following formula:

118 $$IS = 100 - \left(\frac{I\%I_x}{I\%I_0} \times 100\right)$$

119

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

122 The half-maximal effective concentration (EC_{50}), *i.e.* the fungicide concentration inhibiting
123 sporulation of *P. viticola* by 50% compared to the untreated control, was calculated by probit
124 analysis of IS values on log-transformed values of fungicide concentration (SPSS v. 27, IBM Milano,
125 Italy).

126 Sensitivity tests were performed also on 24 sensitive reference isolates of *P. viticola* never exposed
127 to oxathiapiprolin belonging to the collection of the Department of Agricultural and Environmental
128 Sciences (DiSAA, University of Milan). In this case the tested oxathiapiprolin concentration ranged
129 from $1x10^{-6}$-$1x10^{-1}$ mgL^{-1}.

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

The concentration of $4x10^{-1}$ mgL^{-1} oxathiapiprolin was tentatively considered the discriminatory dose for the identification of resistant isolates, based on the observations made in this study (normally no infection occurred from $1x10^{-1}$ mgL^{-1} in sensitive isolates as shown in Supplementary Table 1) and on the information available in the literature, where bulk isolates were considered resistant when their EC_{50} was >1000 times higher than the values recorded by sensitive reference isolates.[15] The discriminatory dose used in this study was 3'000 times higher than the average EC_{50} value of our reference strains ($1.4x10^{-4}$ mgL^{-1}) and >10 times higher than the highest EC_{50} value reported for sensitive *P. viticola* isolates collected in Europe ($3x10^{-2}$ mgL^{-1} oxathiapiprolin)[15]. 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.[3] 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 a stereomicroscope (Zeiss Stemi 305, TiEsseLab, Milano Italy) by 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, the sporangia produced as a consequence of the infection by the single inoculated sporangium were collected and propagated 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 mgL^{-1} oxathiapiprolin to the dose range. The remaining sporangia were stored at -20 °C until DNA extraction.

2.4 DNA extraction and *PvORP1* sequencing

DNA was extracted from suspected resistant strains and from the 24 sensitive reference isolates never exposed to oxathiapiprolin, 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).

A portion (550 bp) of the gene coding for OSBP was amplified by using the following primers synthesized from Microsynth, Balgach, Switzerland: Pv1603F (AAC GTT GCG TAT TCA CAA GA) and Pv1606R (ATC TGT GGG TGT CTT GGA).[15] The amplification of the gene was performed in an Eppendorf Mastercycler Ep (Eppendorf, Milano, Italy) thermocycler on a total volume of 50 µL containing 1x Dream Taq Green PCR Master Mix (Thermo-Fisher Scientific), 0.5 µM of the primers, and 50 ng of DNA. Negative controls (water) were included. Amplification was performed by using the following conditions: 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. Amplified DNA was purified and sequenced (Sanger sequencing) by Eurofins Genomics (Vimodrone, Milano, Italy). Sequencher 5.4.6 software (Gene Codes Corporation, Ann Arbor, MI, USA) was used to compare the predicted amino acid sequence of the *PvORP1* gene from wild-type reference isolates and the oxathiapiprolin resistant mutants. The consensus sequences of two representative sensitive isolates (Pv-0.0 and Pv-0.1) and the two resistant isolates (Pv-16.1 and Pv-16.2) were deposited on GenBank under the accession numbers OP675467 (Pv-0.0), OP675468 (Pv-0.1), OP675469 (Pv-16.1), and OP675470 (Pv-16.2).

3. RESULTS

3.1 Sensitivity assays

The EC_{50} values of the sensitive references ranged from $2x10^{-7}$ to $1.2x10^{-3}$ mgL^{-1} and was equal to $1.4x10^{-4}$ mgL^{-1} on average (Supplementary Table1). No sporulation was observed at oxathiapiprolin concentrations higher than $1x10^{-1}$ mgL^{-1}.

Globally, the disease severity index of the populations on the untreated controls (I%I) ranged from 26.2 to 100 %, with an average value of 74.8 % (Table 2). 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, oxathiapiprolin exhibited a progressive and strong inhibitory effect on *P. viticola* infection at increasing concentrations, as indicated by the high values of IS observed between $4x10^{-5}$ and $4x10^{-1}$ mgL^{-1} of active substance (Table 2; Figure 3). At the lowest concentration ($4x10^{-5}$ mgL^{-1}), more than 20% of the tested samples were already inhibited over 50%. This percentage reached 58% and 93% at $4x10^{-3}$ and $4x10^{-2}$ mgL^{-1} of oxathiapiprolin, respectively. Only three samples (Pv-16, Pv-24 and Pv-26) were able to sporulate at the maximum concentration of 4 10^{-1} mgL^{-1}, and only one of them (Pv-26) showed a IS below 50%.

The EC_{50} profiles calculated from IS values of the populations ranged from a minimum under $4x10^{-5}$ mgL^{-1} to a maximum over $4x10^{-1}$ mgL^{-1}, with an average value of $3.2x10^{-2}$ mgL^{-1} (Table 2). In most of the samples tested, the values calculated were very low, indicating a typical situation of sensitivity. In particular, for seven samples (Pv-05, Pv-12, Pv-14, Pv-15, Pv-20, Pv-22 and Pv-27) the EC_{50} values were below the lowest oxathiapiprolin concentration tested ($4x10^{-5}$ mgL^{-1}). On the other hand, Pv-24 and Pv-26 showed particularly high values: the EC_{50} value coincided with or were higher than, respectively, the maximum concentration of oxathiapiprolin tested ($4x10^{-1}$ mgL^{-1}).

The general situation of sensitivity well described from the values mentioned above, is confirmed by the EC_{50} values very close or, more frequently, below the 0.03 mgL^{-1} threshold reported for oxathiapiprolin-sensitive isolates in Europe(Table 2).[15] The only samples with EC_{50} values $\geq 4x10^{-1}$ mgL^{-1} 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 resistance to oxathiapiprolin with an EC_{50} higher than 4 mgL^{-1} (Table 3), a value that is more than 30'000 times higher than the average EC_{50} value of the sensitive references. Indeed, no substantial decrease in terms of I%I could be appreciated in the $4x10^{-4}$-4 mgL^{-1} concentration range and at the greatest oxathiapiprolin concentration the IS values remained below 30 % (Table 3). As consequence, IS values for each concentration remained very low, reaching a maximum of 27.5 % and 23.8 % respectively at 4 mgL^{-1}.

3.2 Molecular characterization of resistant isolates

Given the resistant phenotypes detected in the sensitivity tests performed, genomic DNA was extracted from Pv-16.1 and Pv-16.2 and the coding region of the *PvORP1* gene was sequenced and compared with that of 24 reference sensitive isolates tested as described above ($EC_{50} < 4x10^{-3}$ mgL^{-1}) belonging to the DiSAA collection sampled before 2020 and therefore never exposed to oxathiapiprolin. The nucleotide and predicted amino acid sequences of *PvORP1* region included among codons 835 and 852 of two representative sensitive strains (Pv-0.0 and Pv-0.1) and the two resistant strains (Pv-16.1 and Pv-16.2) are reported in Figure 4. Compared to the reference sensitive strain Pv-0.0, the analysis of *PvORP1* gene sequence of the sensitive isolate Pv-0.1 isolates revealed four single nucleotide polymorphisms (SNPs) leading to silent mutations (AAC at codon 837, AAA at codon 838, CCT at codon 840 and CTC at codon 851; Figure 4A). These SNPs lead to the same amino acid present in the sensitive reference Pv-0.0 (N837, K838, P840 and L851; Figure 4B). A single SNP was found in the resistant strains at codon 837 (ATT; Figure 4A) changing the amino acid sequence (I837; Figure 4B) of the PvORP1 protein (N837I) (Figure 4). 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.

4. Discussion

In the past years, the costs for research and development of new molecules have escalated, reducing the number of active substances with new modes of action available on the market and making sustainability of efficacy a key issue in the life cycle of an active substance.[14] Due to this limitation, resistance development assumes a significant risk. Indeed, since the multi-site fungicides are progressively disappearing from the market, antiresistance strategies will rely more and more on the combination and/or alternation of single site fungicides, with the risk of selecting strains that are resistant to multiple modes of action. The key element for preserving, at the same time, the efficacy of all the chemical classes available is detecting the shift in sensitivity of the pathogen population before their spreading becomes meaningful.[19] For this reason, it is fundamental 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 and undertake all the actions to manage resistance.

In this study, the new molecule oxathiapiprolin showed excellent activity against most of the samples collected in Italian vineyards, as demonstrated by the EC_{50} values ranging from $8.6x10^{-5}$ to $3.3x10^{-2}$ mgL^{-1} , that are below or very close to the baseline sensitivity range for oxathiapiprolin in European *P. viticola* isolates ($1x10^{-3}$-$3x10^{-2}$ mgL^{-1}).[15] 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 oxathiapiprolin, whereas the data obtained from populations sampled from vineyards treated with a variable number of oxathiapiprolin 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, the EC_{50} values obtained for two of the 29 populations tested (Pv-24 and Pv-26) were over the maximum concentration of $4x10^{-1}$ mgL^{-1} of oxathiapiprolin, which was tentatively considered the discriminatory dose for the identification of resistant isolates according to the results obtained from sensitivity tests on reference strains and the scientific literature.[15] In this context, it is interesting to note that the maximum value of EC_{50} was obtained from the population treated for five consecutive years with oxathiapiprolin before 2019 (Pv-26). Despite the two following and consecutive growing seasons (2019 and 2020) without chemical control, the EC_{50} values of the population were >44 times higher than the average value of sensitive European isolates ($9x10^{-3}$ mgL^{-1}).[15] A possible explanation may be found in the type of assay performed: when dealing with bioassays on populations, a qualitative result can be achieved, *i.e.* an indication on the presence of resistant strains, but not on their amount within the population. It could be therefore possible that resistant strains, selected before 2019, were still present inside the population and determined the high EC_{50} value observed. It would have been interesting to isolate the resistant strains and assess the mutation(s) associated with this phenotype. Unfortunately, for populations Pv-24 and Pv-26 it was not possible to successfully isolate stable single strains from survivors of the 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 resistance to oxathiapiprolin. 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 oxathiapiprolin-resistant mutants in the field might be reduced.[12,15,20,21]

The *P. viticola* strains bearing the mutated *PvORP1* allele (*PvORP1*-837I) isolated from sample Pv-16 exhibited reduced levels of sensitivity to oxathiapiprolin, while reference isolates carrying the wild-type allele (*PvORP1*-837N) expressed a sensitive phenotype and were unable to grow at $4x10^{-1}$ mg/ml of oxathiapiprolin. This suggests a correlation between the presence of this mutation and the resistant phenotype found in sensitivity tests. This amino acidic substitution has already been reported in *P. viticola* Italian field populations by Mboup and collaborators as conferring resistance.[15] Although 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 factors. For the two resistant strains (Pv-16.1 and Pv-16.2) in leaf-disc sensitivity tests we found very low IS values, until the maximum fungicide concentrations (IS=27.5% and 23.8 % at 4 mgL^{-1} respectively), suggesting that individuals carrying the N837I mutation could easily survive and infect *V. vinifera* species in the presence of oxathiapiprolin. 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 oxathiapiprolin) in other oomycetes.[22] This further corroborates the hypothesis that the residue at this position can significantly affect biological activity of OSBP inhibitors. It would be interesting to sequence the whole *OSBP* gene to understand if any other mutation(s) are linked to resistant phenotypes.

5. Conclusion

Results from this work indicate an excellent activity of oxathiapiprolin 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 (high EC_{50}) in biological assays was recorded. Similar observations have been made when investigating oxathiapiprolin efficacy in *P. viticola* and in other oomycetes.[12,15,22] Further studies are needed to confirm the fitness of the *PvORP1*-37I genotypes and investigate the presence of other SNPs

295 associated with the resistant phenotype. Disease management strategy must be carefully planned,
296 taking into consideration the possible spread of this mutation. Given the difficulties in the discovery
297 of new modes of action, the costs of registration of single-site fungicides, the imperative of
298 preserving their effectiveness for as long as possible, the high resistance risk of *P. viticola*,[4,23] and
299 the presence of resistant strains in vineyard, it can be concluded that frequent applications of
300 oxathiapiprolin in the same location should be avoided. Furthermore, the results obtained in this
301 study highlight once again the importance of respecting the principles of antiresistance strategies
302 for single site fungicides such as oxathiapiprolin, that should be adopted in mixture and/or
303 alternation with partner compounds possessing a different mode of action[6,24] and to pay particular
304 attention when using this fungicide in areas characterized by high disease pressure levels, which
305 could be considered to be more prone to the risk of resistance spreading. Finally, the achieved
306 results highlight the necessity of collecting quantitative data (*i.e.* percentage of resistant isolates
307 and EC_{50} values of individual strains) on the pathogen populations. The gathering of these data is
308 hardly possible with the traditional methods of isolation and propagation of *P. viticola*, that are not
309 precise and very time consuming. Hopefully, the recent development of a protocol based on flow
310 cytometry and cell sorting will allow the researchers:[25] i) to isolate, with high precision, single
311 individuals from the population; ii) characterize the isolates for their EC_{50} to accurately estimate
312 resistance factors and discriminatory rates; and iii) to quantify, with high precision, the percentage
313 of resistant sporangia within a population at a discriminatory rate of fungicide.

314

315 **Acknowledgements**

316 The authors wish to thank Stefania Prati and Andrea Giupponi for the management of the plants in
317 the greenhouse, Marco Pravisano for sampling, Jürg Wullschleger for the help in molecular assay
318 development and Fabio Quaglino for the help in the nucleotide sequence submission. The activities

performed within this study fall into the projects “Resistance to mandipropamid and oxathiapiprolin in Italian *Plasmopara viticola* populations” and “Efficacy of antiresistance strategies with mandipropamid and oxathiapiprolin for the control of grapevine downy mildew” funded by Syngenta Crop Protection and Syngenta Italia, respectively.

Conflict of interest

The authors declare no conflicts of interest.

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Tables

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

Sample code	Sampling period and year	Province code	Region	Number of treatments with oxathiapiprolin
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).

405 Table 2: Average disease severity (I%I) on the untreated control, sporulation inhibition (IS) at each
406 oxathiapiprolin concentrations ($4x10^{-5}$-$4x10^{-1}$ mgL^{-1}), and EC_{50} values of *P. viticola* field populations
407 analyzed during the experimental activities. The numbers in brackets represent 95% confidence
408 limits of the EC_{50} values.

Sample code	I%I (%)	IS (%)					EC_{50} (mgL^{-1})
	0	$4x10^{-5}$	$4x10^{-4}$	$4x10^{-3}$	$4x10^{-2}$	$4x10^{-1}$	
Pv-01	50	14.9	33.3	43.3	100	100	$7.1x10^{-03}$ ($1x10^{-3}$; $5.1x10^{-2}$)
Pv-02	66.7	15.0	14.9	85.7	100	100	$7.7x10^{-04}$ ($2.7x10^{-4}$; $2.2x10^{-3}$)
Pv-03	71.4	15.0	14.9	83.3	100	100	$8.7x10^{-04}$ ($2.9x10^{-4}$; $2.6x10^{-3}$)
Pv-04	26.2	15.0	14.9	72.7	100	100	$1.5x10^{-03}$ ($4.4x10^{-4}$; $5x10^{-3}$)
Pv-05	83.3	82.9	94.3	100	100	100	$< 4x10^{-5}$
Pv-06	78.6	33.3	69.7	84.8	100	100	$1.2x10^{-04}$ ($2.9x10^{-5}$; $5.3x10^{-4}$)
Pv-07	85.7	13.9	38.9	50.0	72.2	100	$2.9x10^{-03}$ ($5.5x10^{-4}$; $1.5x10^{-2}$)
Pv-08	95.2	12.5	27.5	45.0	90.0	100	$2x10^{-03}$ ($5.8x10^{-4}$; $7.1x10^{-3}$)
Pv-09	83.3	25.7	28.6	92.1	100	100	$3.4x10^{-04}$ ($1.1x10^{-4}$; $1x10^{-3}$)
Pv-10	100	7.1	23.8	23.8	71.4	100	$1.1x10^{-02}$ ($2.5x10^{-3}$; $4.9x10^{-2}$)
Pv-11	90.5	26.3	36.8	39.5	68.4	100	$4.6x10^{-03}$ ($4x10^{-4}$; $5.3x10^{-2}$)
Pv-12	57.1	75.0	33.3	75.0	91.7	100	$< 4x10^{-5}$
Pv-13	90.5	31.6	28.9	34.2	92.1	100	$1.4x10^{-03}$ ($3x10^{-4}$; $6.6x10^{-3}$)
Pv-14	90.5	76.3	76.3	78.9	100	100	$< 4x10^{-5}$
Pv-15	90.5	42.1	81.6	76.3	76.3	100	$< 4x10^{-5}$
Pv-16	76.2	43.8	9.4	59.4	59.4	71.9	$4.8x10^{-03}$ ($3.1x10^{-4}$; $7.4x10^{-2}$)
Pv-17	92.9	30.8	23.1	38.5	69.2	100	$7x10^{-03}$ ($6.2x10^{-4}$; $7.8x10^{-2}$)
Pv-18	64.3	37.0	51.9	74.1	77.8	100	$2.4x10^{-04}$ ($2.5x10^{-5}$; $2.3x10^{-3}$)
Pv-19	71.4	63.3	46.7	43.3	93.3	100	$8.6x10^{-05}$ ($6.5x10^{-6}$; $1.1x10^{-3}$)
Pv-20	59.5	68.6	68.6	73.5	100	100	$< 4x10^{-5}$
Pv-21	57.1	15.8	21.1	36.8	52.6	100	$3.3x10^{-02}$ ($3.2x10^{-3}$; $3.3x10^{-1}$)
Pv-22	78.6	93.3	93.3	94.9	96.1	100	$< 4x10^{-5}$
Pv-23	50	4.8	19.0	9.5	85.7	100	$8.9x10^{-03}$ ($2.5x10^{-3}$; $3.1x10^{-2}$)
Pv-24	88.1	13.5	10.8	13.5	40.5	54.1	$4x10^{-01}$ ($3.3x10^{-2}$; $5.2x10^{+0}$)
Pv-25	73.8	32.3	19.4	58.1	58.1	100	$6.6x10^{-03}$ ($4.4x10^{-4}$; $9.8x10^{-2}$)
Pv-26	78.6	3.0	6.1	3.0	30.3	33.3	$> 4x10^{-1}$
Pv-27	95.2	72.5	72.5	65.0	82.5	100	$< 4x10^{-5}$
Pv-28	81.0	38.2	50.0	52.6	100	100	$1.1x10^{-03}$ ($1.3x10^{-5}$; $9x10^{-2}$)
Pv-29	45.2	15.8	21.1	36.8	52.6	100	$3.3x10^{-02}$ ($3.2x10^{-3}$; $3.3x10^{-1}$)

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410

411 Table 3: Disease severity (I%I) on the untreated control, sporulation inhibition (IS) at each
412 oxathiapiprolin concentrations ($4x10^{-4}$-4 mgL^{-1}), and EC_{50} values (mgL^{-1}) obtained from sensitivity
413 tests carried out on resistant strains Pv-16.1 and Pv-16.2 isolated from the Pv-16 population during
414 the experimental activities.

Sample code	I%I (%)	IS (%)					EC_{50} (mgL^{-1})
		$4x10^{-4}$	$4x10^{-3}$	$4x10^{-2}$	$4x10^{-1}$	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

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416

Figures

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).

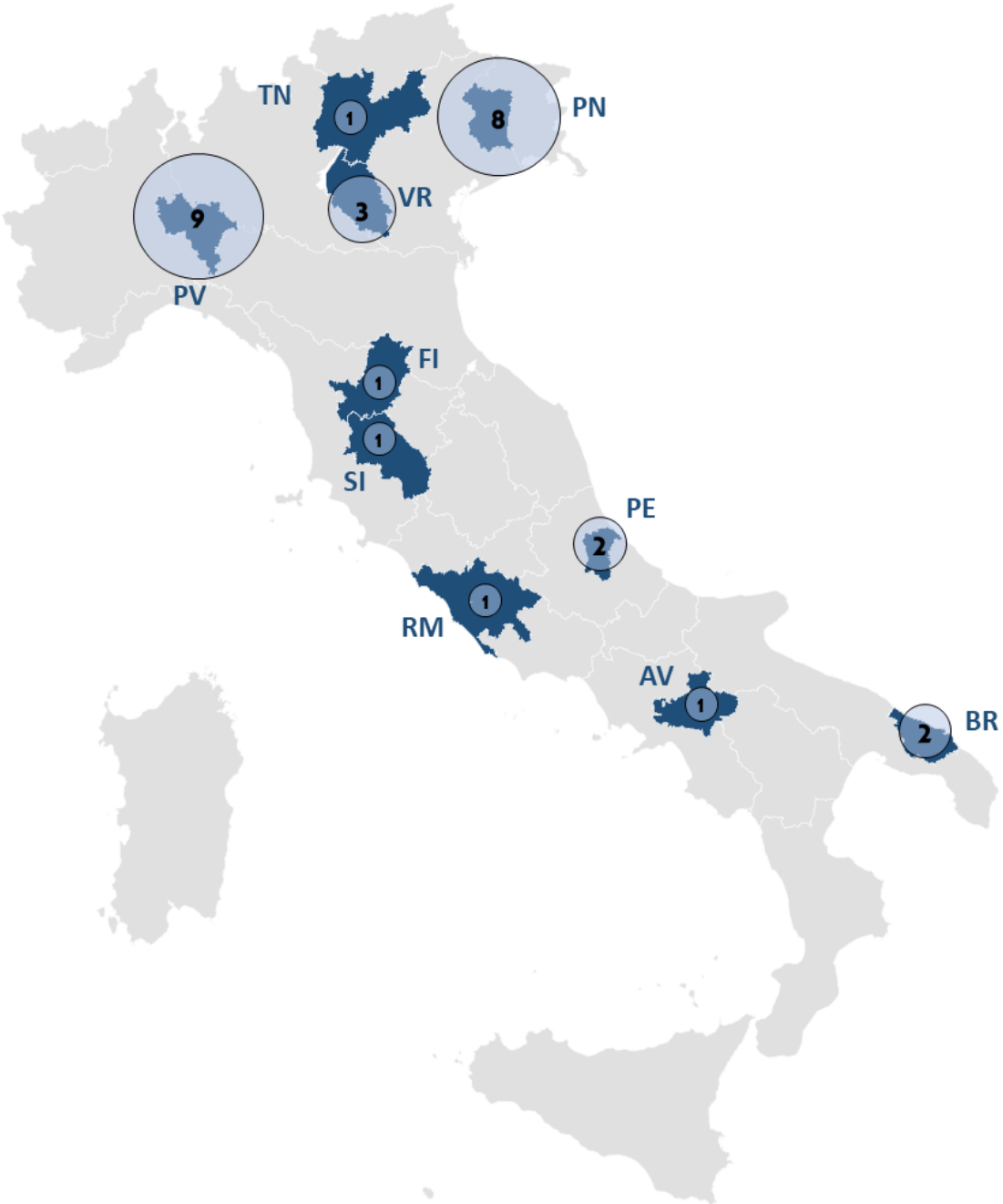

Figure 2: Schematic representation of the sensitivity test performed on the sporangia suspensions obtained from the populations under investigation: six leaf discs originating from six different leaves (cv Pinot-noir) were cut out with a cork borer (A) and placed with the lower side upwards in six different Petri dishes containing moistened paper (B). The leaf discs were sprayed with increasing concentration of oxathiapiprolin (concentrations reported below mgL^{-1}), left to 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).

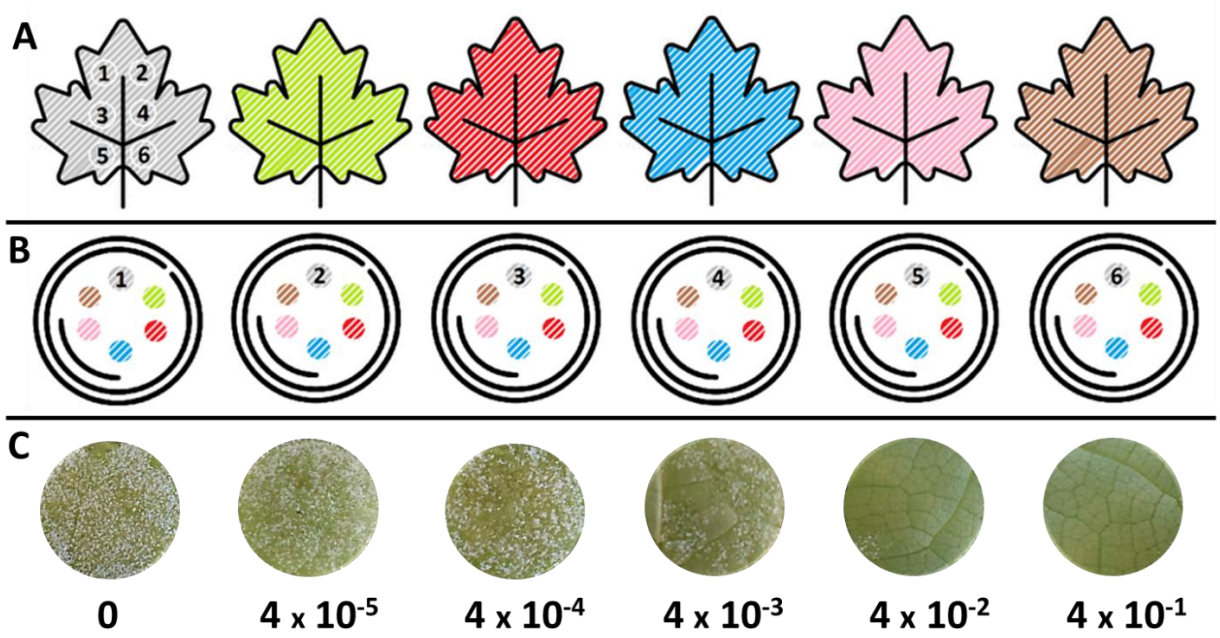

Figure 3. Linear fit of dose-response data, probits of IS values versus the log of the oxathiapiprolin concentrations. Samples are indicated by different colors. Resistant populations are indicated.

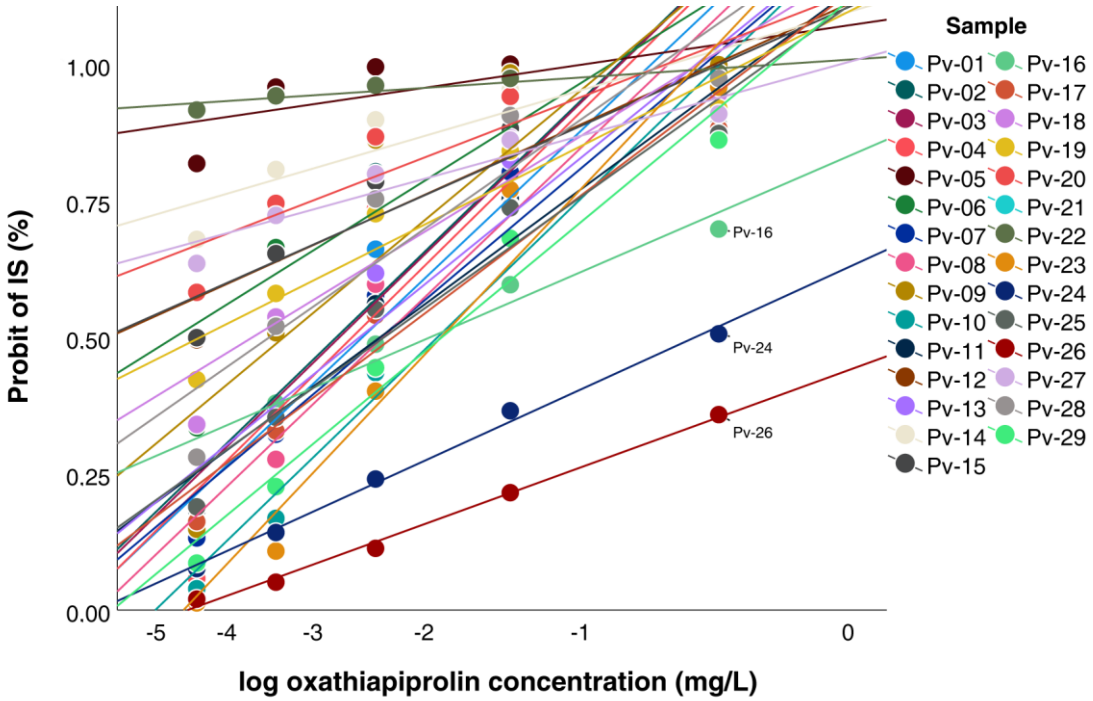

Figure 4: Nucleotide (A) and predicted amino acid (B) sequences of *PvORP1* 835-852 codon region of two sensitive control strains (Pv-0.0 and Pv-0.1) and resistant isolates Pv-16.1 and Pv-16.2. Dots represent nucleotides and amino acids that are identical to those of the sensitive reference Pv-0.0. Letters indicate the nucleotides and amino acids that are different from those of the sensitive reference Pv-0.0. The codon numbers are indicated in bold above the sequences.