Characterization of fungicide sensitivity profiles of *Botrytis cinerea* populations sampled in Lombardy (Northern Italy) and implications for resistance management

Running title: Fungicide resistance profile and management in Lombard *Botrytis cinerea* populations

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ABSTRACT

BACKGROUND

Resistance to fungicides is one of the aspects that have to be considered when planning the treatments to achieve an optimal control of grey mould, caused by *Botrytis cinerea*, in vineyards. In this study, extensive fungicide resistance monitoring was carried out in Northern Italy (Lombardy region) to evaluate several aspects of fungicide resistance (frequency of resistance, effect of field treatments, mechanism of resistance and fitness) on 720 *B. cinerea* strains isolated from 36 vineyards.

RESULTS

Of the characterized strains, 12 % were resistant to a single fungicide class (3 % to the succinate dehydrogenase inhibitor boscalid, 4 % to the anilinopyrimidine cyprodinil; 5 % to the phenylpyrrole fludioxonil; 0.1 % to the ketoreductase inhibitor fenhexamid) and 0.8 % to two fungicide classes contemporaneously. Resistance was associated with mutations reported in the literature for boscalid

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(H272Y/R) and fenhexamid (P238S or I232M). Two new mutations in *sdhC* (A187F) and in *sdhD* (I189L) could be related to boscalid resistance. Strains resistant to fludioxonil did not show any known mutations. No significant differences were found in the fitness of sensitive and resistant strains.

CONCLUSION

Overall, field populations of *B. cinerea* showed a relatively low frequency of resistance, but the geographical distribution of resistance, genetic mechanisms of resistance and fitness of resistant strains suggest that management of resistance should be implemented, at local and regional levels. Particular attention should be given to the fungicide sprays planned before veraison, since they seem to be associated with a higher frequency of resistant strains in vineyards.

KEYWORDS: fungicide resistance; anti-resistance strategies; disease control; Botrytis rot;

1. INTRODUCTION

Grey mould is one of the main diseases affecting grapevines and grapes both in the field and postharvest.¹⁻⁴

Disease management in vineyards strongly relies on the application of fungicides with a single-site mode of action in the great majority of cases.⁵ Among the most used single-site fungicide classes employed in vineyards, listed according to the cross-resistant behaviour established by FRAC (www.frac.info), are: succinate dehydrogenase inhibitors (SDHIs), including boscalid; anilinopyrimidines (APs), including cyprodinil, mepanipyrim and pyrimethanil; phenylpyrroles (PPs) such as fludioxonil; and ketoreductase inhibitors (KRI), such as the hydroxylanilide fenhexamid. Resistance to members of the individual classes or simultaneous resistance to different classes (multidrug resistance, MDR) has been reported in *Botrytis cinerea* (Pers.) strains isolated in vineyards located in France⁶, Germany^{7,8}, Italy⁹⁻¹³, United States¹⁴, Chile¹⁵, Australia¹⁶ and New Zealand¹⁷. Fungicide resistance in *B. cinerea* is associated with point mutations at the gene encoding for the fungicide target and the overexpression of drug efflux transporters (ABC

transporters).⁶ The latter mechanism is mainly related to MDR, a phenomenon that can potentially complicate the management of resistance in the open field.

An optimized management of resistance can be achieved by gathering information on the disease management strategy and characterizing *B. cinerea* populations for resistance. This allows the implementation of anti-resistance strategies, by delaying of the selection pressure of a fungicide class if needed. A thorough comprehension of the resistance status of a pathogen population should, however, consider other important aspects of the phenomenon, which are related to the mechanisms of resistance and to the fitness of resistant strains. Resistance may be a consequence of genetic changes in the fungal strains that are stable and inheritable¹⁸, and may lead to the spread and survival of resistant strains. But, if a mutation conferring resistance to a fungicide class has a pleiotropic effect, where decreased sensitivity is associated with a decreased fitness, resistance in practice (a loss of disease control due to the severe presence of resistant strains in the field) could be delayed or may not emerge.¹⁹ Reduced fitness was indeed reported in *B. cinerea* strains resistant to fenhexamid, that showed a lower growth rate and production of sclerotia and conidia in comparison with wild-type strains.²⁰

The Lombardy region, located in the central part of Northern Italy, is an important vine-growing region with great geographic variability, where grapevines are grown in mountain areas (*e.g.* Sondrio province), hills (*e.g.* Pavia and Mantova provinces) and plains (*e.g.* Brescia province). Grey mould control mainly relies on fungicide applications, that are carried out according to phenological and meteorological criteria. Generally, four treatments are applied per season²¹: the first two before veraison, *i.e.* at the end of flowering (A) and at bunch closure (B); the other two at veraison (C), *i.e.* at the onset of ripening, and three weeks before harvest (D). If meteorological conditions are less conducive to the disease, it is possible that only two treatments may be needed. Despite a relatively low number of fungicide sprays being carried out per

season, the limited number of fungicide classes available for disease management, all single-sites, potentially favours the selection of resistant strains.

A previous study, principally aiming at characterizing the genetic variability of 317 *B. cinerea* strains isolated from 36 vineyards in Lombardy, showed that isolates resistant to cyprodinil, fludioxonil and boscalid were present at low frequency in the region.¹² In this paper, 720 *B. cinerea* strains were isolated from the same vineyards with the objective of evaluating: (i) the frequency of resistant strains of *B. cinerea* isolated from vineyards treated with members of SDHI, AP, PP and KRI fungicides; (ii) the effect of the fungicide treatments carried out in vineyards on the selection of resistant strains; (iii) the genetic basis of resistance; and (iv) the fitness of resistant and sensitive strains.

2. MATERIALS AND METHODS

2.1 Sampling procedure and fungicide sensitivity assays

A total number of 720 strains of *B. cinerea* were obtained by sampling at harvest symptomatic berries from 36 vineyards located in the most important viticultural areas of Lombardy, located in the provinces of Sondrio (SO), Brescia (BS), Mantova (MN) and Pavia (PV) (supporting information Table S1). Twenty symptomatic bunches were collected per vineyard and a single monoconidial strain was isolated per organ.¹⁰ With the exception of the vineyards no. 5, 11, 12, 17, 22, 30 and 35, the vineyards were treated at least with an active substance belonging to the SHDI, AP, PP or KRI fungicide classes, during the sampling season or in previous years. Sensitivity to boscalid, cyprodinil, fenhexamid and fludioxonil was assessed by using an automated method.¹²

2.2 Molecular characterization of the strains

The presence of mutations related to fungicide resistance was investigated on 30 representative strains which were resistant to boscalid (18 strains), fludioxonil (5 strains), cyprodinil and fludioxonil (2 strains) and fenhexamid (3 strains) or fully sensitive to the four fungicides (2 strains). The target genes or transcription

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factors screened for resistance were: succinate dehydrogenase gene subunits B (*SdhB*), C (*SdhC*) and D (*SdhD*) for boscalid resistance²³; C3-keto-reductase (*erg 27*) gene for fenhexamid resistance²³; and transcription factor *mrr1* for resistance to fludioxonil and, more in general, to resistance to multiple fungicides.²⁴ The molecular mechanism of AP resistance was not known, therefore strains could not be genotyped for cyprodinil resistance. DNA was extracted¹⁰, quantified at the spectrophotometer (Nanodrop, ND1000, ThermoFisher Scientific, Milano, Italy) and diluted with nuclease-free water to a final concentration of 50 ng/μL. PCR was performed in a Eppendorf Mastercycler Ep (Eppendorf, Milano, Italy) thermocycler in 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 1, and 200 ng of DNA. Negative controls were included. Amplification was performed by using the following conditions: an initial denaturation cycle at 95° C for 2 minutes; 35 cycles of denaturation at 95° C for 30 s, annealing at the temperatures reported in Table 1 for 30 s, extension at 72° C for 1 min; and a final extension at 72° C for 5 min. Amplified DNA was purified and sequenced by Eurofins Genomics (Vimodrone, Milano, Italy).

2.3 Characterization of the strains for fitness components

Mycelial growth, colony morphology and conidia production were assessed on 75 *B. cinerea* strains: 8 resistant to boscalid, 15 resistant to cyprodinil, 23 resistant to fludioxonil and 29 sensitive to all the active substances. Due to the low frequency, strains resistant to fenhexamid were excluded from these analyses. Mycelial growth was assessed on Malt Agar (MA, Oxoid, Basingstoke, UK) at three different temperatures (15, 20, 25 °C) and on PDA (Potato Dextrose Agar, Oxoid) at 20 °C in the dark. Mycelial plugs (Ø, 5 mm) of actively growing colonies were excised with a sterile cork borer from the edges of the actively growing cultures and transferred to the centre of Petri dishes (9 cm diameter) containing the above cited media. Radial growth measurements were daily carried out between one and four days after inoculation (dai=day after inoculation).²⁵ For each combination of strain and cultural conditions, three replicates were prepared.

Two perpendicular colony diameters per dish were measured and the mean colony diameter was calculated.

The morphology of *B. cinerea* strains was evaluated at 5, 10 and 20 days after incubation at 20 °C on PDA.

The strains were classified in four categories, depending on the aspect of the vegetative and reproductive structures (Figure 1). Category 1 encompassed strains with a uniformly distributed mycelium with sporulation (Figure 1a), category 2 those with mycelium distributed in masses and sporulation (Figure 1b), category 3 those without sporulation (Figure 1c) and category 4 the strains with sclerotia (Figure 1d).

Conidial concentration was estimated five and ten days after incubation of *B. cinerea* strains at 20 °C on PDA. Conidia were collected from each plate with a sterile loop, resuspended in 1 mL of 20 % glycerol-water solution and counted at the microscope in a Kova counting grid (Hycor Biomedical Inc., Garden Grove, California, USA) following the manufacturer's instructions.

Pathogenicity of the 82 *B. cinerea* strains was assessed on *V. vinifera* leaves (cv Pinot noir) and berries (cv Italia).¹⁰ Three leaf discs (2.5 cm diameter) were cut from three different leaves, placed in Petri dishes (9 cm diameter) and inoculated with a mycelial plug (5 mm diameter), excised from the edges of an actively growing *B. cinerea* colony. Six surface-sterilized berries were placed in a humid chamber, wounded with a sterile inoculation needle and inoculated with a mycelial plug as previously described. Each test was performed in three replicates. The infected organs were incubated at 20 °C and scored for the symptomatic area from 7 to 11 dai for leaves and from 3 to 7 dai for berries.¹⁰ The percentage infection index (I%)²⁶ determined at each time point was used to calculate the area under the disease progress curve (AUDPC).²⁷⁻

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2.4 Statistical analysis

The EC₅₀, which is the effective concentration of fungicide that reduces mycelial growth by 50% compared to the untreated control, was computed as a parameter of a log-normal dose-response model defined as follows:

$$y = c + (d - c)\{\Phi[b(\log(x) - \log(e))]\}$$

where y is the response variable associated to the inhibition level, c is the lower limit, d is the higher limit, Φ is the distribution function for Normal distribution, b is the model's slope, x is the fungicide's rate and e is the ED₅₀.³⁰ The sensitivity profile of each strain was determined according to the resistance factor (RF).¹²

The Mann-Whitney U test was used to evaluate: 1) differences between the EC₅₀ values recorded for boscalid, cyprodinil, fludioxonil and fenhexamid, grouping the strains according to RF values (lower or higher than 10) and the application or not of the respective fungicide classes (SDHI, AP, PP and KRI); 2) the effect of the application of the fungicide class during the four years preceding sampling on the percentage of resistant strains; 3) differences in the distribution of the AUDPC values of resistant and sensitive strains.

The Kruskal-Wallis test was used for evaluating differences in: 1) distribution of the percentage of resistant strains according to the phenological stage of the treatment (samples treated both before and after veraison were discarded from this analysis), and geographic origin of the strains; 2) distribution of conidia concentration (conidia/mL) of sensitive and resistant strains; 3) distribution of AUDPC values of sensitive and resistant strains on leaves and berries. Rank-transformed data were used for all non-parametric statistics. χ^2 test was performed on the colony morphology data. Significance level was set at 0.05.

The effects of temperature, growth substrate and fungicides' resistance phenotype on growth dynamics of *B. cinerea* strains were investigated by Linear Mixed effect Model (LMM) as follows:

$$y_{ij} = \eta_{ij} = \beta_0 + \beta_T x_T + \beta_d x_d + \beta_j + \beta_{Ph} + \varepsilon_{ij}$$

where y_{ij} is the *B. cinerea* colony's diameter for the i -th strain growing on the j -th growth substrate at certain time d (days) and temperature T (°C); η_{ij} is the linear predictor where β_0 is the intercept given by the sum of

the fixed intercept parameter b_0 and its strain-dependent random component u_{0i} so that $\theta_0 = b_0 + u_{0i}$, with $u_{0i} \sim N(\mu_i, \sigma^2_i)$; θ_T is the temperature's slope given by the sum of the fixed parameter b_T and its strain-dependent random component u_{Ti} so that $\theta_T = b_T + u_{Ti}$, with $u_{Ti} \sim N(\mu_i, \sigma^2_i)$, whereas x_T is the temperature in °C; θ_d is the growth time slope given by the sum of the fixed parameter b_d and its strain-dependent random component u_{di} so that $\theta_d = b_d + u_{di}$, with $u_{di} \sim N(\mu_i, \sigma^2_i)$, whereas x_d is the growth time in days; θ_j is the growth substrate effect with $j=(MA, PDA)$ where MA is the reference factor level; θ_{Ph} is the fungicide resistance phenotype effect with $Ph=(S,B,C,FL)$ where S is the reference factor level (S =sensitive phenotype; B =boscalid resistant phenotype; C =cyprodinil resistant phenotype; FL =fludioxonil resistant phenotype); finally, ε_{ij} is the error term assumed to be normal with 0 mean and σ^2 variance, hence $\varepsilon_{ij} \sim N(0, \sigma^2)$. The choice of using strains as subjects (*i.e.*: the main experimental unit for the random effects' definition) is aimed at accounting for the use of a fairly large number of *B. cinerea* strains chosen randomly among different resistance phenotypes during the experiment. The consequent heterogeneity of errors was addressed by accounting for non-constant variances according to the exponential variance's function suggested by Pinheiro and Bates (2006).³¹ Moreover, the complicated correlation structure due to the multiple strains' dynamic response to temperature (*i.e.*: each strain might grow either more slowly or more quickly under different temperatures) was addressed by allowing the LMM to have an unconstrained positive definite variance–covariance matrix computed according to the log-Cholesky parameterization.³² The model fit was assessed by Observed vs Simulated (OS) simple linear regression using the simulated *B. cinerea* colonies' diameters as an independent variable (*i.e.*: the *B. cinerea* colonies' diameter estimates obtained by the LMM) and the measured *B. cinerea* colonies' diameters as a dependent variable, in order to compute the pseudo- R^2 (nearest to 1 is better) and evaluate the capacity of the simulated data to explain the real measured data.³³ The t -test for such simple linear regression intercepts ($H_0: a=0$) and slopes ($H_0: b=1$) were also reported. LMM fit by Restricted Maximum-Likelihood estimation method (REML) and the ANOVA table reporting its overall fixed effect

significance were respectively performed by the nlme() and the anova.lme() functions both implemented in the nlme library, R.3.5 package, whilst the simple linear regression for the goodness of fit's assessment was performed by lm() function implemented in the stats library, R.3.5 package. Fenhexamid resistant strains were excluded from this analysis due to their low frequency.

3. RESULTS

3.1 Sensitivity of *Botrytis cinerea* strains

Of the 720 *B. cinerea* strains, 640 were characterized for resistance to boscalid and fenhexamid, 618 for resistance to cyprodinil, and 639 for resistance to fludioxonil. For all fungicide classes tested, the EC₅₀ values (Figure 2) recorded by individual strains grouped according to the resistance factor (lower or higher than 10) showed significantly different distributions. *B. cinerea* strains were, therefore, classified as sensitive or resistant to the individual fungicides based on resistance factors (RF<10 for sensitivity; RF>10 for resistance). Based on the data distribution, it was moreover possible to identify the concentrations able to discriminate between sensitive and resistant strains for each fungicide: EC₅₀ values higher than 1.2 mg/L characterized strains resistant to boscalid; EC₅₀ values higher than 3.2 mg/L were recorded by strains resistant to cyprodinil; EC₅₀ values higher than 0.1 mg/L discriminated strains resistant to fludioxonil from the sensitive ones; and finally, EC₅₀ values higher than 0.5 mg/L characterized strains resistant to fenhexamid.

The great majority of the strains (>95 %) showed a sensitive phenotype in relation to the four chemicals tested, apart from those isolated from SO that showed sensitivity to fludioxonil in 89 % of the cases (Table 2). The percentage of resistant strains ranged between 1 and 6 % for boscalid, between 3 and 5 % for cyprodinil, and between 0 and 0.4 % for fenhexamid with no differences among provinces. The percentage of strains resistant to fludioxonil ranged from 2 to 11 % and significant differences were found among the values recorded in the different provinces, with SO being the province with the highest frequency of

resistant strains. Five strains showed simultaneous resistance to cyprodinil and either boscalid (strains n. 19, 29 and 674) or fludioxonil (strains n. 397 and 473).

Even if the median values were close in absolute terms (Table 3), the distribution of EC₅₀ values recorded by the strains isolated from treated vineyards were significantly different from those of the strains isolated from untreated vineyards for all fungicides. The percentages of resistant strains were significantly higher in vineyards where the fungicides were applied before veraison (mean=7.3 %; median 5.5 %) than after this phenological stage (mean= 4.1 %; median=0 %). According to statistical analysis, the percentage of resistant strains was significantly higher in vineyards where fungicides were applied in the past four years (mean=6.2 %; median= 5%) than where they had not been applied (mean=3.2 %; median=0 %).

3.2 Molecular characterization of the strains

The mutations associated with resistance found in the present study are listed in Table 4. The isolates resistant to boscalid showed two mutations at codon 272 of *sdhB* gene: H272R (44 % of the isolates) or H272Y (17% of the strains). Two non-synonymous mutations, not present in the literature nor in the sensitive strains analysed in this study, were found at *sdhC* (A187F) and *sdhD* (I189L) genes of three resistant strains. Interestingly, strain no. 632 showed the simultaneous presence of three mutations: H272R at *sdhB*, A187F at *sdhC* and I189L at *sdhD* genes. Two out of the three strains resistant to fenhexamid showed the P238S mutation at *erg 27* gene, whereas the third strain had the I232M mutation. None of the strains resistant to fludioxonil nor MDR phenotypes showed point mutations nor leucine deletion at codon 497 of the transcription factor *mrr1*.

3.3 Characterization of the strains for fitness parameters

The results obtained on mycelial growth on MA and PDA at different temperatures, conidia concentrations and pathogenicity on berries and leaves (expressed as AUDPC) are reported in supporting information Table S2.

Concerning mycelial growth, the observed and simulated (OS) simple linear regression fitted to assess the LMM's goodness of fit showed a Pseudo- R^2 about 0.90, hence 90% of the total variance in the observed data was explained by the simulated ones estimated by the LMM (Figure 3A). Such estimates appeared unbiased and consistent with the observed data, as the OS simple linear regression parameter's t test for intercept a and for the slope b were both non-significant, whence the t tests' null hypotheses for a ($H_0: a=0$) and b ($H_0: b=1$) were both accepted. Indeed, glancing at t tests' p-values (P_t), it can be noticed that the probability of being wrong in rejecting the t tests' null hypothesis was about 25.1% for a and about 94.2% for b . Moreover, the LMM's residuals approximately approached a normal distribution (Figure 3B). The LMM's parameters' p-values (P_t) were lower than 0.0001 for the intercept, the temperature's slope, the growth time slope and the growth substrate, thus the corresponding fixed effects were all highly significant. By contrast, the p -value obtained by the ANOVA solution for the LMM's fixed effects (P_F) shows that the overall fungicide resistance phenotype (FR phenotype) effect was not significant. Indeed, the fungicide resistance phenotype effect parameters did not show significant differences between sensitive phenotypes (S) and each of resistant phenotypes (B, C and FL).

A significant association was found between colony morphology and time in both sensitive and resistant strains. At 5 dai, most of the sensitive strains showed a mycelium in masses (52 %) or with sporulation (35 %) and few (13 %) showed a mycelium without sporulation (Figure 4A). At ten dai, the main phenotype was the sclerotial type (52 %) followed by mycelium in masses (41 %) and with sporulation (7 %). At 20 dai, most of the strains showed a sclerotial type phenotype (82 %) and few had a mycelium with sporulation (18 %). Resistant strains followed the same trend of sensitive strains with some differences. Strains resistant to boscalid (Figure 4B) and fludioxonil (Figure 4D) at 10 dai showed a low frequency of sclerotial phenotypes (13 and 30 % respectively). While all boscalid-resistant strains switched to a sclerotial phenotype at 20 dai,

only 70 % of fludioxonil resistant isolates produced sclerotia. Cyprodinil resistant strains (Figure 4C) were the only ones showing a mycelium without sporulation at 10 and 20 dai, even if at low frequency (7 %). Conidia production of the strains grouped according with the resistant phenotype ranged from 3.7 to 5.1×10^5 conidia/mL at 5 dai and from 4.7 to 6.5×10^5 conidia/mL at 10 dai (supporting information Table S2). No significant differences in conidia concentration were found among sensitive and resistant strains at both 5 and 10 dai.

As regards pathogenicity tests, the average AUDPC values of the strains grouped according with the resistant phenotype ranged from 110 to 145 on berries and from 153 and 159 on leaves (supporting information Table S2). No significant differences were found among the distributions of the AUDPC values of the strains grouped according to their resistant phenotype on both berries and leaves nor between AUDPC values on leaves and berries within resistance phenotypes.

4. DISCUSSION

The results obtained on the characterization of *B. cinerea* population for resistance showed that the resistance factor value higher than 10 used in the present study was able to discriminate between sensitive and resistant strains. A relatively low frequency of resistant strains (0.1-5 %) was found in the region, as described in previous studies.^{10,12} This could be attributed to the limited number of fungicide applications (1-2 per season, in general) carried out on wine grapes. The limitation in the number of fungicide sprays per season can, in fact, slow down the selection of resistant strains.³⁴ The percentage of strains resistant to boscalid (2-6 %) is in line with that reported in other studies^{11,15,17} and lower than that reported for German vineyards, where the frequency of boscalid-resistant strains reached up to 70 %.⁸ Resistance to SDHI is of relatively recent discovery, and could probably be related to the increased use of the class.⁸ Therefore, particular attention should be taken in monitoring its evolution in the field. Resistance to APs has been frequently reported in grapevine fields, where the percentage of resistant isolates can be lower than 10

%^{12,15,17}, as found in the present study, or reach values ranging from 30 to 80 %.^{7-9,11} Resistance to APs was detected early in the 2000s and is particularly widespread⁵, however it proved to be unstable in absence of selection pressure: this could help to explain the wide variability in the diffusion of resistant strains found in the literature. Resistance to PPs^{8,9,12} and KRIs^{7,8,13,15,16} in vineyards has been less frequently reported in the literature than resistance to members of the first two classes. While almost full sensitivity to the KRI member fenhexamid was found in the present study, the relatively high frequency of strains resistant to fludioxonil (5 % on average) highlights the need to further investigate the phenomenon and analyse the disease management strategies adopted in vineyards to decrease the selection pressure of the class towards insensitive strains, that at present seems to be one of the main issues in the region. Differences in the frequencies of strains resistant to fludioxonil were found at the province level, where a single province (SO) was responsible for the higher presence (11 %) of resistant strains compared to the others. This could be explained by the history of fungicide treatments in this province, where 44 % of the vineyards were sprayed with PP in the four previous years, whereas the percentage dropped to 30 % in the other provinces. Statistical analysis, indeed, showed that the application of the fungicide classes, both during the year of sampling and in previous years, had an influence, even if of little absolute value, on the selection of resistant strains in *B. cinerea* populations. This is not surprising, since the emergence of resistance is an evolutionary process based on the fungicide modulation that involves progressive changes in the gene pool of the fungal population leading to a large enough resistant subpopulation.³⁴⁻³⁷ Interestingly, the phenological stage at which the fungicides were applied showed a role in shaping the pathogen population: the frequency of resistant strains was, in fact, higher in vineyards where the fungicides were applied before than after veraison. This could indicate that the earlier in the season is the selection of resistant strains, the higher is their diffusion during the ripening stages, when the polycyclic epidemic starts.² In temperate regions, *B. cinerea* epidemiology on grape starts in spring, with the colonization of flower tissues by the

pathogen. Subsequently, *B. cinerea* enters a latent phase or a saprophytic over-summering phase until ripening, when the mature berries are easily colonized by the pathogen and repeated cycles of fruit infection occur.² Therefore, if resistant strains were selected by the fungicide early in the season, they could have a higher possibility of spreading at ripening, when the berries are more susceptible to the pathogen and weather conditions are favourable to disease establishment, thanks to the moderate temperatures and higher rainfall frequency of the late summer season.³⁸ Further investigations are however needed to confirm these results.

Multidrug resistance, *i.e.* the simultaneous resistance of a strain to fungicides with different modes of action, is a well-known phenomenon in *B. cinerea*.³⁹⁻⁴² MDR strains resistant to 2-5 different fungicide classes (APs, PPs, SDHIs, QoIs, benzimidazoles and dicarboximides) have been found at high frequency on table grape fields located in Southern Italy, where up to 8 fungicide treatments are carried out per season.⁹ In this study, MDR strains, with low sensitivity to cyprodinil and boscalid or cyprodinil and fludioxonil, were sporadically found, probably due to the lower frequency of fungicide treatments performed in the region, where only wine grapes are cultivated. The detection of MDR strains is, however, of great concern for grey mould control in the region, where formulations containing APs and PPs in mixtures are frequently applied. Resistance to boscalid has been related to point mutations at codon 272 (H272Y/R/L/V), 225 (P225F/L) or at codon 230 (N230I) of the gene encoding for the SDHB subunit of the succinate dehydrogenase complex.^{9,43} The molecular characterization of boscalid-resistant strains showed that two point mutations in *sdhB* gene, H272R and H272Y, were the most frequently detected in Lombardy, as reported in the literature for moderately resistant strains.^{5,37} Two mutations, A187F at *sdhC* and I189L at *sdhD* genes, not previously reported in the literature, were moreover found alone or in combination with mutations at *sdhB*. Since these mutations were found in a sporadic number of strains, further investigations are needed to investigate their role in resistance to boscalid. Indeed, although previous studies reported mutations of

sdhC in *B. cinerea* strawberry isolates, strict correlation with resistance to boscalid and fluopyram could not be found.⁴⁴ Resistance to fenhexamid has been associated with point mutations in *erg27* gene leading to an alternative aminoacid at codons 412 (F412I/S/V), 408 (Y408H), 170 (G170R), 210 (A210G) or the deletion of proline at codon 298.^{45,46} None of the strains analysed in this study showed the F412S/I/V mutation found in highly resistant strains⁵, but fenhexamid resistant strains showed P238S and I232M mutations in the *erg27* gene. P238S mutation has been described in the literature as a silent mutation not necessarily associated with resistant phenotypes^{46,47} as well as associated with highly (HydR3⁺ phenotypes) and moderately (HydR3⁻ phenotypes) resistant Chilean isolates.⁴⁸ Analogously, the I232M mutation was reported in moderately resistant isolates (HydR3⁻ phenotypes).⁴⁴ Indeed the EC₅₀ values of the strains that showed RF>10 ranged from 0.6 to 1.1 mg/L of fenhexamid, values that are compatible with those previously reported for moderately to slightly resistant strains (0.2-2 mg/L range).⁴⁷ Resistance to fludioxonil and, more in general, resistance to multiple fungicides in *B. cinerea* strains has been associated with mutations in the transcription factor *mrr1*, leading to the overexpression of *atrB* and, as a consequence, to the overexpression of drug efflux transporters.^{24,41} In particular, a point mutation at codon 632 of *mrr1* (Li, 2015) and a 3-bp deletion at codon 497 in *mrr1*, leading to the absence of a leucine (L) in the Mrr1 protein and correlated with MDR1h isolates²⁴, were found. The fludioxonil-resistant strains analysed in the present study showed EC₅₀ values ranging from 0.2 to 1.3 mg/L of active ingredient, values that were previously described for MDR1 and MDR1h isolates of *B. cinerea*.⁴⁹ MDR1 strains showed resistance to fludioxonil and cyprodinil due to the overexpression of *atrB*, and MDR1h phenotypes are a MDR1 variant with higher levels of resistance to the two fungicides.²⁴ As in isolates of *Botrytis fragariae*, a newly described species⁸, also in this study no mutations in *mrr1* were found in *B. cinerea* strains resistant to fludioxonil.⁴⁹ Taken together, the results obtained by the molecular characterization suggest that the strains resistant to boscalid and fenhexamid isolated in Lombardy possess a stable and inheritable

mechanism of resistance, that is based on point mutations at the genes encoding for target proteins, even if associated with moderately or variably resistant phenotypes. Concerning fludioxonil, the resistant strains analysed in the present study could possess an alternative or unknown mechanism of resistance than that described in the literature, as hypothesized for *B. fragariae*.⁴⁹

One of the key points in the management of fungicide resistance is the evaluation of the fitness, that can be defined as the ability of a fungus to grow, reproduce and survive in the environment⁵⁰, of resistant strains. Knowledge of the fitness of a phenotype can help in making hypotheses on the evolution of a population and, in the specific case of fungicide resistance, can help in estimating the risk of further diffusion and persistence of resistant strains.⁵¹ Fungicide-resistant strains with no fitness penalties can, in fact, spread and persist in the population even in the absence of selection pressure, complicating the disease management by causing reduced fungicide performance once the fungicide is applied. To evaluate whether resistance was associated with fitness penalties, several parameters, related to growth on synthetic media, conidia production, colony morphology and pathogenicity^{20,50,52,53}, have been measured in sensitive and resistant strains. Statistical analysis showed that strains resistant to boscalid, cyprodinil and fludioxonil did not show significantly reduced abilities to grow on MA and PDA and to produce conidia in comparison with sensitive isolates. Looking at the morphology of the colonies it was evident that both sensitive and resistant phenotypes showed an increasing trend towards sclerotia differentiation from 10 to 20 dai. While half of the sensitive isolates formed sclerotia already at 10 dai, strains resistant to boscalid and fludioxonil reached and exceeded this percentage at 20 dai. This could indicate a slight delay in the production of sclerotia by these phenotypes that, however, formed sclerotia for the most part of the isolates at 20 dai. Sclerotia are formed between 6 and 28 dai⁵³ and are necessary for the pathogen survival during unfavorable conditions⁵⁴, such as the absence of the host during the winter, even if they can be one of the ways the pathogen adopts for surviving in vineyards.² Statistical analysis of AUDPC values indicated that sensitive and

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resistant phenotypes are characterized by analogous abilities to colonize leaves and berries. Since berries represent a more suitable substrate for *B. cinerea* growth than leaves, due to the higher content in pectin and sugars¹⁰, the absence of differences in the ability to colonize the two different organs within resistant phenotypes suggests that the strains generally possess a high virulence. The results obtained by the evaluation of the fitness components suggest that sensitive and resistant strains are characterized by analogous saprophytic aptitudes, as demonstrated by growth on nutritive media⁵⁵, pathogenicity and conidia production, and a slight delay in the differentiation of survival structures but only in the case of boscalid and fludioxonil resistant strains.

5. CONCLUSION

The relatively low diffusion of strains resistant to the four chemical classes employed in Lombardy suggests that fungicide resistance can be managed with appropriate anti-resistance strategies. However, the absence of fitness costs of resistant strains indicates that these isolates could easily diffuse and survive in the pathogen population. Therefore, the disease management strategy must be carefully planned, taking into consideration also the history of the vineyard, not only at regional level but also at a local level, as demonstrated by the higher presence of fludioxonil-resistant strains in the province of Sondrio. In this regard, the modulation of the fungicide treatments should be carefully considered, since it seemed to have a strong influence on the selection of resistant strains, not only within one season but over different seasons. The active substances should be chosen and applied in mixtures and/or alternation according to the resistance profiles of the population, in order to decrease the selection pressure of the disease management strategy. The higher risk of selecting resistant strains associated with the treatments carried out before veraison observed in the present studies should be moreover taken into consideration in further studies.

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Table 1. List of the primers used for genotyping resistance to boscalid, fludioxonil and fenhexamid, sequence of the primers, annealing temperatures (T_a), direction and reference.

Fungicide	Target	Primer	Sequence (5'-3')	T_a (°C)	Strand	Reference
boscalid	<i>sdhB</i>	KES429	ATGGCTGCTCTCCGCACAG	56	fw	Grabke and Stammler, 2015
		KES167	TGCTATCTCATCAAGCCCCCT		rv	
	<i>sdhC</i>	KES622	ATGTTTTACAGAGAGCAACTCAAC	54	fw	
		KES623	CTACAAGAAAGCAACCAACGC		rv	
	<i>sdhD</i>	KES667	ATGGCTTCATTCATCAACCATC	52	fw	
		KES668	TTATGCGCGCCAAATTCTTTTG		rv	
fludioxonil	<i>mrr1</i>	Mrr1-spez-F	TATCGGTCTTGCAGTCCGC	55	fw	Leroch <i>et al.</i> , 2013
		Mrr1-Pira	CCACCACAATCTTGATCATTGGGATCAGAACCTGC		rv	
fenhexamid	<i>erg27</i>	KES2004	TGCTCCCTATAGTTTCAACC	55	fw	Grabke and Stammler, 2015
		KES2005	AGCAAAAGAGTTTGAACAGG		rv	
		erg27_Beg	TGGGATTACCACCATGGGAGACAAGTG		fw	
		erg2000up	TCGGAGGGTTTGGCTTGTTTTG		rv	

Table 2. Average percentages of *Botrytis cinerea* strains resistant to boscalid, cyprodinil, fludioxonil and fenhexamid in the four provinces of Lombardy (BS, MN, PV and SO) and in the overall region.

Fungicide	Location				Region
	BS	MN	PV	SO	
Boscalid	6	2	3	1	3
Cyprodinil	5	4	4	3	4
Fludioxonil	2	2	4	11	5

Fenhexamid	0.4	0	0	0	0.1
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Table 3. Number, percentage and median distribution of the EC₅₀ values of strains divided according to the application (yes) or not (no) of each fungicide class (SDHI, AP, PP and KRI) in vineyard.

Fungicide class	Application in vineyard	Strain N.	Strain %	Median
SDHI	yes	500	78	0.61
	no	140	22	0.53
AP	yes	206	33	0.02
	no	412	67	0.01
PP	yes	357	56	0.03
	no	282	44	0.01
KRI	yes	569	89	0.03
	no	71	11	0.02

Table 4. List of the mutations found in *Botrytis cinerea* isolates resistant to boscalid and fenhexamid. Strains with more than one mutation are underlined.

Strain number	Resistance	Gene	Mutation
361,362,719	boscalid	<i>sdhB</i>	H272Y
363, 377, 379, 576, <u>632</u> , 636, 668, <u>674</u> ,	boscalid	<i>sdhB</i>	H272R
<u>632</u>	boscalid	<i>sdhC</i>	A187F
614, <u>632</u> , <u>674</u>	boscalid	<i>sdhD</i>	I189L
135, 167	fenhexamid	<i>erg27</i>	P238S
278	fenhexamid	<i>erg27</i>	I232M