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Panebianco, A; Castello, I; Cirvilleri, G; Perrone, G; Epifani, F; Ferrara, M; Polizzi, G; Walters, DR; Vitale, A

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Detection of *Botrytis cinerea* field isolates with multiple fungicide resistance from table grape in Sicily

Anna Panebianco [a], Ivana Castello [a], Gabriella Cirvilleri [a], Giancarlo Perrone [b], Filomena Epifani [b], Massimo Ferrara [b], Giancarlo Polizzi [a], Dale R. Walters [c], Alessandro Vitale [a,] *

[a] Department of Agriculture, Food and Environment,, University of Catania, Catania 95123, Italy

[b] Institute of Sciences of Food Production National Research Council (ISPA-CNR) Bari 70126, Italy

[c] Crop and Soil Systems Research Group, Scotland's Rural College, Edinburgh, United Kingdom EH9 3JG, UK

ABSTRACT

During 2009-2013, 302 single-spore isolates of *Botrytis cinerea* were collected from vineyards located in the most important site of table grape production in Sicily, recognized by the European Community as Protected Geographical Indication (PGI) 'Mazzarrone grape'. In preliminary studies, all isolates were tested *in vitro* for their sensitivity to six fungicides belonging to the following groups: benzimidazoles, dicarboximides, anilinopyrimidines, succinate dehydrogenase inhibitors, hydroxyanilides and phenylpyrroles. In these tests, 45.7% of the isolates were found to be resistant to at least one fungicide. Specific resistance to pyrimethanil was found in 30.8% of the isolates, whereas 13.9, 10.3 and 7.6% of the isolates exhibited resistance to carbendazim, iprodione and boscalid, respectively. No isolates resistant to fenhexamid and fludioxonil were detected within our dataset of *B. cinerea* isolates. However, 30 *B. cinerea* isolates possessed multiple resistance to two or more fungicides. In detail, 8 isolates were simultaneously resistant to four fungicides, whereas 5 and 17 isolates were resistant to three and two fungicides, respectively. For boscalid, 11/23 of isolates showing *in vitro* resistance possessed a mutation at the *Sdh*B gene, whereas all isolates resistant to carbendazim and iprodione possessed mutations at β-tubulin and BcOS1 histidine kinase genes, respectively. Accordingly, these fungicides failed to control grey mould infections caused by resistant or reduced sensitivity isolates on grape berries and grapevine leaves whereas the sensitive isolates were effectively managed by all fungicides applied at label rates. This study represents the first report of *B. cinerea* field isolates resistant and/or with simultaneous resistance to several botryticides from table grape vineyards in Sicily. Therefore, current strategies for fungicide resistance management of *B. cinerea* could be negatively affected in future.

Keywords:

Botrytis cinerea

multiple fungicide resistance

table grape

boscalid

* Corresponding author. Section of Plant Pathology, Department of Agriculture, Food and Envinroment, University of Catania, Santa Sofia 100, Catania 95123 IT.

E-mail address: alevital@unict.it (A. Vitale).

1. Introduction

Grey mould, caused by *Botrytis cinerea* Pers.: Fr., is a major fungal disease of table grape (*Vitis vinifera* L.) worldwide. This pathogen is responsible for heavy losses in one of the most important Italian areas of table grape production known as 'Mazzarrone grape', an area that is recognized by the European Community with the label 'Protected Geographical Indication' (PGI, Reg. CE 617/2003). Grey mould represents the most serious threat for this typical production since the grape harvesting is usually performed up to late December when the climatic conditions occurring in vineyards are favourable for disease development. Although cultural practices which increase air movement and decrease humidity levels can help to manage botrytis bunch rot in vineyards, effective strategies rely mainly on preventive treatments of different botryticides. Grey mould symptoms generally become prominent in vineyards after bunch closure (Holz and Volkmann, 2002); thus two-to-five spray applications of site-specific compounds are usually performed at the bunch pre-closure stage, at the beginning of and during berry ripening. Over the last 35 years, several molecules belonging to methyl benzimidazole carbammates (MBCs), dicarboximides, anilinopyrimidines (APs), hydroxyanilides, phenylpyrroles and more recently, succinate dehydrogenase inhibitors (SDHIs), have been used in this area. Unfortunately, the selective pressure exerted by chemical control against this 'high risk' pathogen induces development of fungicide-resistant isolates. The major mechanism of resistance in *B. cinerea* is

mutation in the genes encoding the target site protein causing reduced fungicide binding. These modifications, often determining the 'specific resistance' towards a single or one class of fungicide, were first detected for anti-microtubule fungicides (e.g. MBCs), and successively verified for dicarboximides, hydroxyanilides, strobilurins, and SDHIs (Fillinger et al., 2008; Leroux et al., 2002, 2010). Besides specific resistances, multiple fungicide resistance has also been recently detected in French and German vineyards, but it usually exhibits considerable resistance levels towards several classes of botryticides that are mediated by a single gene (Kretschmer et al., 2009). In the past, fungicide resistance within some *B. cinerea* populations was reported on several crops (Amiri et al., 2013; Baroffio et al., 2003; Brent and Hollomon, 2007a; Myresiotis et al., 2007; Weber, 2011). Field resistance of *B. cinerea* to various fungicides has also been detected in vineyards worldwide, resulting in poor fungicide efficacy (Beever et al., 1989; Latorre et al., 2002; Latorre and Torres, 2012; Leroux, 2007; Sergeeva et al., 2002). The use of site-specific fungicides to control high resistance risk pathogens, such as *B. cinerea*, may further increase the development of field resistance (Brent and Hollomon, 2007b). Therefore, continuous monitoring of fungicide resistance is crucial following the first detection of resistant genotypes in vineyards to ensure that adequate anti-resistance strategies are implemented to prevent or delay breakdown of fungicide efficacy.

For these reasons, and related to the lack of information on resistance of *B. cinerea* to these fungicides in Sicily, the aim of this research was to provide the first data on sensitivity to MBCs, dicarboximides, APs, hydroxyanilides, phenylpyrroles and SDHIs within a population of *B. cinerea* isolates, obtained from table grape vineyards within the production area of 'Mazzarrone grape'. Specifically, the objectives of this study were (i) to determine *in vitro* sensitivity to boscalid, carbendazim, fenhexamid, fludioxonil, iprodione and pyrimethanil and their relative *in vivo* performance using detached grape berry and grapevine leaf assays, (ii) to identify point mutations in field isolates resistant to different fungicides, and (iii) to investigate the presence of isolates with multiple fungicide resistance within a population of *B. cinerea*.

2. Materials and methods

2.1. Fungal isolates

In total, 302 isolates of *B. cinerea* were collected over the five-year period between 2009 and 2013 from 15 commercial table grape vineyards located in Ragusa (Acate, Comiso and Chiaramonte Gulfi) and Catania (Caltagirone, Licodia Eubea, Mazzarrone) provinces, constituting the entire 'Mazzarrone district' (recently surveyed for other phytopathological studies) (Vitale et al., 2012). The entire table grape production district has a history of severe infections of botrytis bunch rot. Therefore, treatments with a range of fungicides, including MBCs, dicarboximides, phenylpyrroles, hydroxyanilides, APs, the SDHI-boscalid and other botryticides have been used. In the last ten years, the most frequently used fungicides in this area were Scala® [active ingredient (a.i.) pyrimethanil] and Switch® (a.i. cyprodinil + fludioxonil) (up to two applications per season), Cantus® (a.i. boscalid) and Teldor Plus® (a.i. fenhexamid) (one application per season). Thiophanate-methyl (Enovit Metil®) and iprodione (Rovral Plus®) have only occasionally been included in fungicide programme against grey mould of grape of the surveyed vineyards.

Isolations were made from single infected grapes taken at different places of each vineyard by transferring a small amount of mycelium and/or spores from an infected berry (i.e. one isolate per grape) with a sterile needle onto Petri dishes containing potato dextrose agar (PDA; Oxoid, Basingstoke, UK). Single-conidial isolates were obtained on water agar (WA; Oxoid, UK) at 25°C for 8–16 h. Isolates thus obtained were stored on PDA slants at 4°C.

2.2. Fungicides

All isolates were tested for their sensitivity to six active ingredients [a.i.(s)] belonging to different chemical groups (Table 1). Since thiophanate-methyl showed a lesser persistence than carbendazim on artificial media (PPDB Pesticide Property DataBase: http://sitem.herts.ac.uk/aeru/ppdb/en/atoz.htm), carbendazim was used in *in vitro* assays whereas thiophanate-methyl was employed for grape bioassays. All a.i.(s) were prepared from their commercial formulations. Stock solutions of fungicides were prepared in sterilized distilled water (SDW).

Table 1

Chemical features, trade names, rates and FRAC code (http://www.frac.info) of fungicides used in the *Botrytis cinerea* experiments.

FRAC Code	Active Ingredient	Trade name (Formulation)	Chemical Group	Field Rate	Manufacturer
7	Boscalid	Cantus (WG)[c]	Pyridine-carboxamides	1.0 kg ha^{-1}	BASF SE, Ludwigshafen, Germany
1	Carbendazim[a]	Bavistin (SC)	Benzimidazoles (MBC)	-	BASF SE, Ludwigshafen, Germany
1	Thiop-methyl[b]	Enovit Metil (WG)	Thiophanates (MBC)	1.5 kg ha^{-1}	SIPCAM SpA, Salerano on Lambro, Italy
12	Fludioxonil	Geoxe (WG)	Phenylpyrroles	1.0 kg ha^{-1}	Syngenta Crop Protection, Monthey, Switzerland
17	Fenhexamid	Teldor Plus (SC)	Hydroxyanilides	1.5 L ha^{-1}	Bayer Crop Science AG, Dormagen, Germany
2	Iprodione	Rovral Plus (SC)	Dicarboximides	1.5 L ha^{-1}	BASF Agri-Production, Genay Cedex, France
9	Pyrimethanil	Scala (SC)	Anilino-pyrimidines	2.0 L ha^{-1}	Bayer Crop Science, Wolfenbüttel, Germany

[a] Used in *in vitro* assays. Bavistin is not registered for the use on grape.

[b] Used in bioassays.

[c] WG, water dispersible granule; SC, suspension concentrate.

2.3. Fungicide sensitivity

The sensitivity of *B. cinerea* isolates to fungicides was assessed by measuring radial growth on agar plates amended with different concentrations of a.i.(s). All fungicides were tested on PDA except for pyrimethanil and boscalid, which were tested on a minimal medium containing 10 g of glucose, 1.5 g of K_2HPO_4, 2 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 2 g yeast extract and 12.5 g of agar (Oxoid) per liter of distilled H_2O (Hu et al., 2011; Myresiotis et al., 2007, 2008). Yeast extract was not added in the sensitivity assay for pyrimethanil (Myresiotis et al., 2007). Autoclaved agar media were cooled to about 45°C and amended with appropriate volumes of the fungicide stock solutions to obtain the following a.i. concentrations: 0.05, 0.5, 1, 5, 10, 20 and 50 μg mL^{-1} for boscalid; 0.01, 0.1, 1, 10 and 100 μg mL^{-1} for carbendazim; 0.001, 0.005, 0.01, 0.05, 0.1 and 1 μg mL^{-1} for fenhexamid and fludioxonil; 0.1, 1, 5, 10 and 20 μg mL^{-1} for iprodione and 0.01, 0.05, 0.1, 1, 5, 10 and 50 μg mL^{-1} for pyrimethanil. Unamended media plates served as controls. Mycelium plugs, cut from the edge of an actively growing culture on agar media, were placed upside down on the centre of each fungicide-amended or control dish. Dishes were incubated at 20 °C in darkness for 3–5 days. For each concentration, three plates were used and colony diameter was measured in two perpendicular directions, subtracting the original diameter of the mycelium plug (6 mm) for the calculated value. These assays were performed twice. Radial growth on each plate was measured and the raw data from three replicates used to calculate growth reduction (GR) = [1 – (radius in amended plates/radius of control plates)] × 100. The effective fungicide concentration to inhibit 50% of mycelial growth (EC_{50}) was calculated for each isolate by linear regressions of the mycelial growth reductions versus the $\log_{10}$ transformation of the fungicide concentrations. Frequency distributions of the isolates between the intervals of EC_{50} values were established.

On the basis of the literature, pathogen sensitivity to the fungicides was initially related to discriminatory doses as follows: 1 µg mL^{-1} for carbendazim, iprodione, boscalid and pyrimethanil, and 0.1 µg mL^{-1} for fenhexamid and fludioxonil (Baroffio et al., 2003; De Miccolis Angelini et al., 2010; Faretra and Pollastro, 1991; Latorre and Torres, 2012; Leroux et al., 1999; Myresiotis et al., 2007; Yourman and Jeffers, 1999; Zhang et al., 2007). Only for boscalid, the authors subsequently considered a Resistance Factor (RF) = 5 (the ratio of the EC50 value for a boscalid-resistant isolate relative to the EC50 value for a highly boscalid-sensitive isolate) as distinguishing sensitive from resistant isolates.

2.4. Molecular analysis

To identify the mutations correlated with resistance to boscalid, the complete coding sequence of the *sdhB* subunit (complete succinate dehydrogenase iron sulphur protein gene) of representative *B. cinerea* isolates, selected on the basis of phenotypic sensitivity to the fungicide (sensitive or resistant) in *in vitro* assays, was compared to the corresponding gene sequence of the reference sensitive strain T4 of *Botryotinia fuckeliana* (GenBank accession no. AY726618.1). The resistance to the MBC "carbendazim" was identified by comparing the coding sequences of β-tubulin of the tested *B. cinerea* strains to the corresponding gene sequence of the reference sensitive strain SAS56 (GenBank accession no. Z69263.2). The same approach was also used to identify mutations correlated to resistance to iprodione; here, the coding sequences of *BcOS1* genes (coding for histidine kinase) of the *B. cinerea* strains were compared to reference sensitive strain Bc56 (GenBank accession no. AB064962.1). Genomic DNA was extracted and purified from mycelia of *B. cinerea* isolates grown on PDA for 5 days in darkness. Mycelia were harvested and washed in SDW, frozen in liquid nitrogen and lyophilized. DNA from each isolate was extracted using the kit Wizard® Magnetic DNA Purification System for Food (Promega, Madison, USA). The purified DNA was eluted in a final volume of 100 µL and checked by

electrophoresis on 0.8% agarose gel. The concentration and purity of DNA extracted was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific Instruments). Based on the known complete sequence of the β-tubulin gene in *B. cinerea* (GenBank accession no. U27198), the PCR primer pair Bcb-F (5'-CACTGAGGGTGCTGAGCTTGT-3') and Bcb-R (5'-AGCGGCCATCATGTTCTTA-3') was designed to amplify the β-tubulin gene fragment containing codons 198 and 200 relevant to identifying the isolates resistant to benzimidazoles (Zhang et al., 2010). The primers B1189/2346F (5'-CCCACTACCCCACACCTATG-3') and B1189/2346R (5'-ACAAGCATCGGTTTTGGAAC-3') were used to amplify the *sdhB* sequence and to determine the resistance of isolates to boscalid (De Miccolis Angelini et al., 2010). Two specific primers were designed (Banno et al., 2008), Dicarb 1082_F (5'-CCCAGGGTGAGATACTCCAA-3') and Dicarb 1828_R (5'-AGTTTCTGGCCATGGTGTTC-3'), suitable to amplify 747 bp that includes the possible mutations found among codons 365–369. The PCR products were purified with Exosap-it (Affimetrix, CA), a mixture of exonuclease I and alkaline phosphatase used to remove unincorporated dNTPs and primers present in the PCR products, and then they were sequenced using BigDye Terminator V3.1 Cycle Sequencing Ready Reaction Kit (Applera, USA). Only for the BcOS1 the amplicon of expected size was purified by agarose gel electrophoresis and excised from agarose gel using spin columns (NucleoSpin® Gel and PCR Clean-up - Macherey Nagel). Sequencing was performed on an ABI PRISM 3730 Genetic Analyzer (Applera) and the amplicon sequences were aligned using BioNumerics 5.1 (Applied Maths, Belgium) software to locate and identify the base changes.

2.5. Assays on grape berries

The efficacy of the fungicides used in this study for the control of *B. cinerea* was determined on detached grape berries cv. 'Italia' as previously reported (Parafati et al., 2015; Vitale et al.,

submitted). At least two sensitive and four to five resistant isolates or isolates with reduced sensitivity to each fungicide were selected according to both *in vitro* and molecular data. Single detached berries with pedicel were surface disinfected with 2% of NaOCl for 2 min and rinsed twice in SDW. After drying, four wounds (1-2 mm deep) were made with a sterile hypodermic needle before being sprayed with a fungicide suspension. Boscalid, fenhexamid and fludioxonil a.i.(s) were used at 500 mg L^{-1}, iprodione at 750 mg L^{-1}, pyrimethanil at 800 mg L^{-1}, and thiophanate-methyl at 1 g L^{-1}, respectively. These dosages reflect the rates recommended for botrytis bunch rot of table grape for six commercial formulations registered in Italy (Table 1). Thirty berries were used for each treatment (10 berries/replicate) and placed in a cage containing an aluminum tray at the bottom of which a thin layer of water was poured to maintain high relative humidity (RH). Treatments were applied with a hand-pump until berries were thoroughly wet. After 6 h, the berries were inoculated by placing a 20 µL drop of the conidial suspension (1-2 × 10^5 conidia mL^{-1}) obtained by flooding 10 day-old sporulating cultures on PDA plates with SDW at the surface of the wounds. Berries were placed in separate rows (40 mm apart) on expanded metal sheets in clear plastic-covered cages. The same number of berries sprayed with SDW served as control. For each isolate, lesion diameter (severity of decay) on each berry and the number of infected berries per treatment (disease incidence) were recovered after 6 days of incubation at 24–25 °C. Severity of grey mould decay was calculated both on treated and control grape berries determining its relative reduction of botrytis rot (control efficacy %). The experiment was performed twice.

2.6. Assays on grapevine leaves

As above reported, the same *B. cinerea* isolates were inoculated on potted 3-week-old grapevine cuttings (*Vitis vinifera* L.) cv. Italia to evaluate the fungicide efficacy in controlling grey mould leaf decay. The grapevine cuttings were previously grown in a chamber at 25 °C and

70% RH with a photoperiod of 16 h. Subsequently, the plants were sprayed to run-off with the fungicide suspensions at the same rates used in the previous assay. After two hours, the leaves of these plants were inoculated with selected *B. cinerea* isolates. Six mycelial plugs removed from the margin of the colonies growing on PDA were placed on the upper surface of each leaf. Three leaves (i.e. three replicates) were used for each isolate. The control plants were sprayed with SDW and then inoculated with PDA plugs containing *B. cinerea* mycelium. To create favorable conditions for infection, inoculated plants were covered with plastic bags and incubated in the growth chamber at 25 °C with a photoperiod of 16 h and high RH (90–95%). The disease incidence and diameters of the developing lesions were measured 4 days after inoculation. Severity of grey mould infections was compared between treated and control grape leaves and relative reductions were determined for each isolate. The experiment was carried out twice.

2.7. Data analysis

Data from *in vitro* and *in vivo* sensitivity tests from repeated experiments were combined; one-way analyses of variance (ANOVA) of EC_{50} and grey mould decay values from two experiments showed that they did not differ statistically ($P > 0.05$).

All *in vivo* data were subjected to ANOVA according to parametric or nonparametric approaches (Statistica 10, Statsoft Inc., Tulsa, OK). All percentage data were transformed using arcsine ($\sin^{-1}$ square root x) prior to statistical analysis. The percentage of infected sites caused by pathogen on fungicide-treated grape berries and grapevine leaves are shown and compared among different isolates of *B. cinerea* isolates according to Fisher's least significant difference test ($P < 0.05$ and 0.01). Data on reduction of lesion diameter caused by *B. cinerea* on grape berries and grapevine leaves were analyzed within each tested isolate for pairwise combinations (treated and control) using the non-parametric Mann-Whitney test.

253 **3. Results**

254

255 *3.1. Pathogen sensitivity to fungicides*

256

257 The EC_{50} range and frequency of resistant isolates for all fungicides are reported in Table 2.
258 The 302 isolates of *B. cinerea* tested showed a roughly normal distribution of EC_{50} values to
259 boscalid. Among them, 254 (84.1%) were classified as highly sensitive to boscalid (HS), since
260 their EC_{50} < 1 µg mL^{-1}, whereas 25 isolates (8.3%) had EC_{50} values between 1 and 4.99 µg mL^{-1}
261 and were considered as sensitive (S) isolates. The values for most of these isolates fell within
262 0.1–0.49 µg mL^{-1} range (Fig. 1–A). The remaining 23 isolates (7.6%) grew on media
263 supplemented with boscalid concentrations of 5 µg mL^{-1} or more (Table 2). In detail, 12 isolates
264 (4%) had EC_{50} values ranging from 5 to 19.99 µg mL^{-1} (RF values within 5–20 range) and were
265 considered as reduced sensitivity (RS) phenotypes, three (1%) had EC_{50} between 20 and 49.99
266 µg mL^{-1} and eight (2.6%) isolates had EC_{50} values higher than 50 µg mL^{-1} (Fig. 1–A). Isolates
267 with EC_{50} falling within the 20–50 µg mL^{-1} range and having EC_{50} > 50 µg mL^{-1} were
268 considered resistant (R) and highly resistant (HR) isolates, respectively.

269 Similarly, 260 isolates (86.1%) were found to be sensitive to carbendazim, having EC_{50} values
270 less than 1 µg mL^{-1} (Table 2). The remaining 42 isolates (13.9%), having EC_{50} higher than 100
271 µg mL^{-1}, were considered resistant (Fig. 1–B).

272 Most of *B. cinerea* isolates tested (89.7%) were found to be sensitive to iprodione with a
273 roughly normal distribution (Fig. 1–C). The EC_{50} values for these isolates ranged from 0.1 to
274 0.69 µg mL^{-1} with the highest frequency of values falling within 0.2–0.29 µg mL^{-1}. Otherwise,
275 31 isolates (10.3%) showed resistance to iprodione and grew on media amended with fungicide
276 concentrations higher than 1 µg mL^{-1} (Table 2, Fig. 1–C).

277 About 69.2% of the isolates were found sensitive to pyrimethanil (Fig. 1–D), with EC_{50} values
278 between 0.03 and 0.86 µg mL^{-1}. For this fungicide, a high frequency of resistant isolates (30.8%)

was detected within the *B. cinerea* population since they grew on media amended with pyrimethanil at concentrations higher than 1 µg mL^{-1} (Table 2). Overall, 15.2% of isolates exhibited an EC_{50} value within the 1.0–1.99 µg mL^{-1} range, 7.0% showed EC_{50} values between 2.0 and 4.99 µg mL^{-1} and 8.6% had an EC_{50} value higher than 5 µg mL^{-1} (Fig. 1–D).

No isolates resistant to fenhexamid and fludioxonil were found within the *B. cinerea* population. The frequency distributions of their EC_{50} values were roughly unimodal curves and these data are shown in Fig. 1–E and Fig. 1–F, respectively.

Table 2

Sensitivity of *Botrytis cinerea* isolates from table grape to different tested fungicides.

Fungicide	EC_{50} (µg mL^{-1})		No. of isolates		Resistance
	Sensitive	Resistant	Sensitive	Resistant	frequency (%) [a]
Boscalid	0.01 – 1.81	5.05 – > 50	279	23	7.6
Carbendazim	0.02 – 0.30	> 100	260	42	13.9
Fludioxonil	0.0001 – 0.04	–	302	–	–
Fenhexamid	0.0002 – 0.09	–	302	–	–
Iprodione	0.10 – 0.69	1.16 – 9.27	271	31	10.3
Pyrimethanil	0.03 – 0.86	1.09 – 41.42	209	93	30.8

[a] Resistance frequency values were determined based on discriminatory concentrations of 0.1 µg mL^{-1} for fenhexamid and fludioxonil, and 1 µg mL^{-1} for boscalid, carbendazim, iprodione and pyrimethanil.

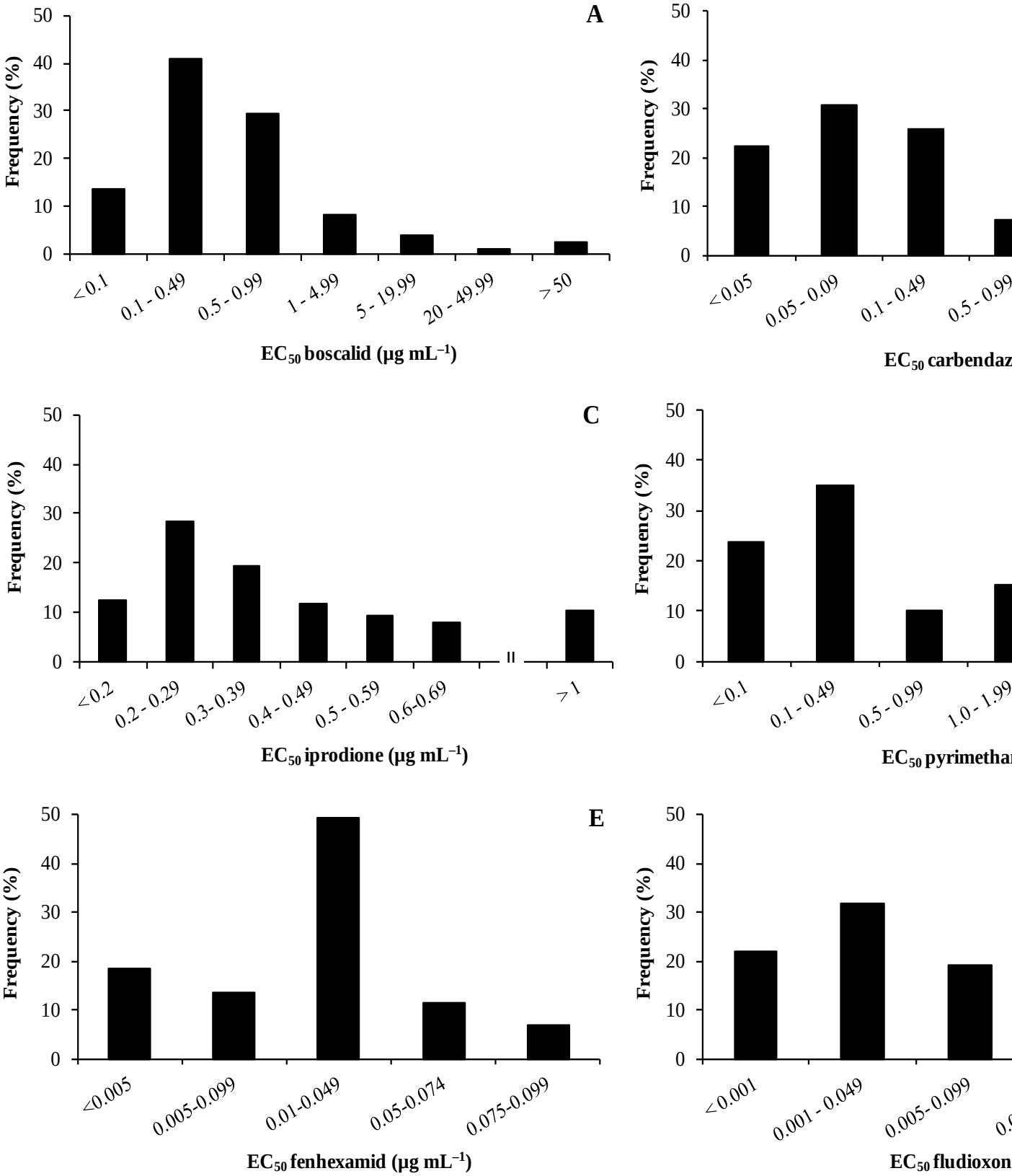

Fig. 1. Frequency distribution of EC_{50} values for boscalid, carbendazim, iprodione, pyrimethanil, fenhexamid and fludioxonil among 302 isolates of *Botrytis cinerea* collected from different vineyards in Sicily.

297

298 *3.2. Multiple resistance among fungicides*

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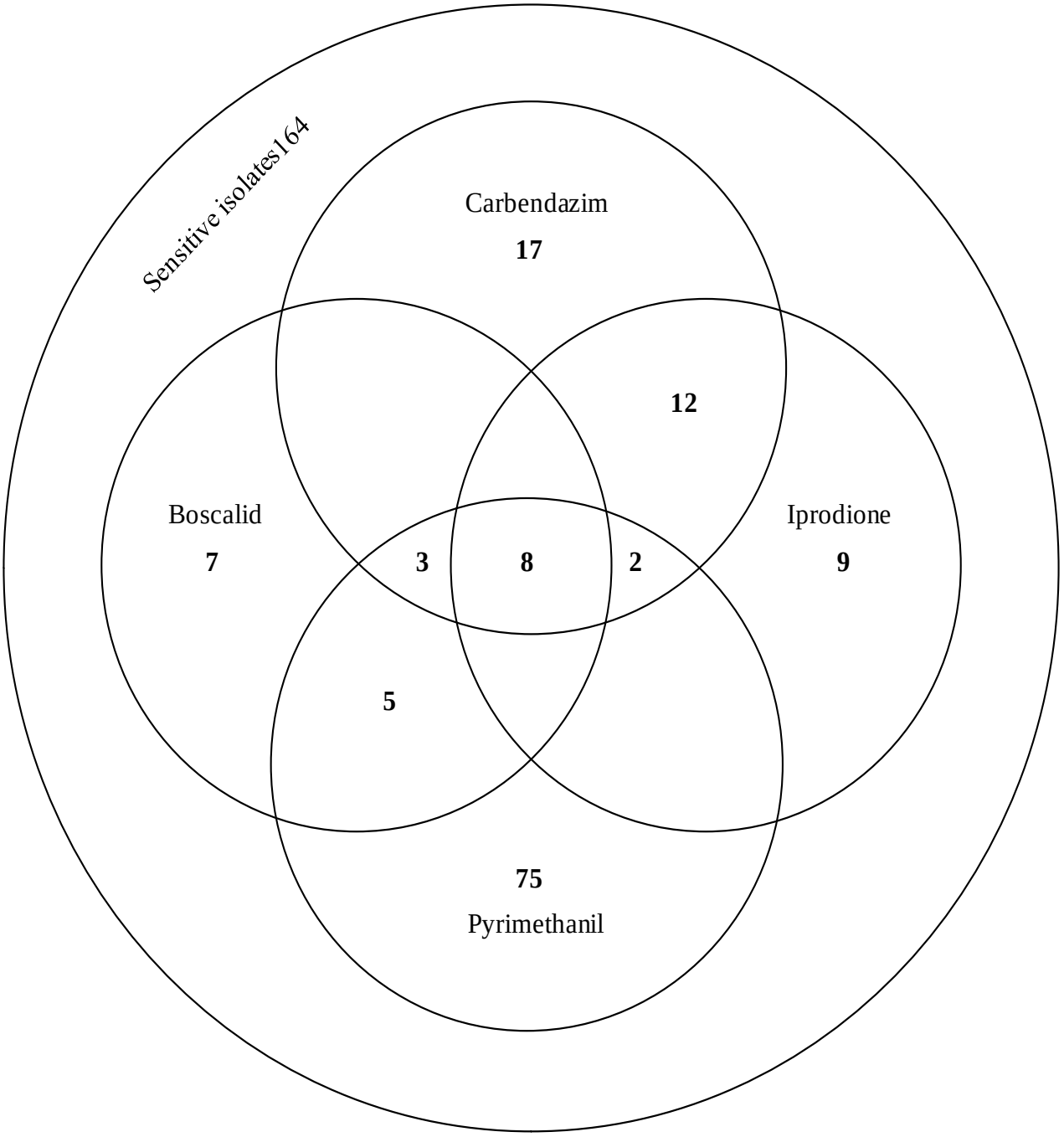
300 A Venn diagram of sensitivity and resistance to fungicides showed that, among all isolates, 30
301 isolates exhibited simultaneous *in vitro* resistance to two or more fungicides (Fig. 2). In detail,
302 five isolates were simultaneously resistant to both boscalid and pyrimethanil and twelve to both
303 carbendazim and iprodione. Three isolates were simultaneously resistant to boscalid,
304 carbendazim and pyrimethanil, two were simultaneously resistant to carbendazim, iprodione and
305 pyrimethanil, whereas eight isolates were simultaneously resistant to boscalid, carbendazim,
306 iprodione and pyrimethanil (Fig. 2, Table 3).

307

308



309

310 **Fig. 2.** Venn diagram indicating simultaneous resistance to boscalid, carbendazim, iprodione and
311 pyrimethanil among 302 *Botrytis cinerea* isolates collected from table grape vineyards during 2009-2013.
312 EC_{50} values higher than 1 μg mL^{-1} (carbendazim, iprodione and pyrimethanil) and 5 μg mL^{-1} (boscalid,
313 RF = 5) classified isolates as resistant and/or with reduced sensitivity to fungicides. The large circle
314 represents the full set of 302 isolates tested for fungicide sensitivity. Each of four smaller circles
315 represents the set of isolates with reduced sensitivity to the corresponding active ingredients. The

316 intersections among different circles indicates 4 subgroups that were simultaneously resistant to more than
317 one fungicide.
318

Isolate	Municipality	Province	Boscalid	Carbendazim	Iprodione	Pyrimethanil
2010						
SR1, SR5	Licodia Eubea	Catania	R [a]	S [a]	S	R
MZ2.1, MZ2.2	Chiaramonte G.	Ragusa	S	R	R	S
MZ2.11	Chiaramonte G	Ragusa	R	R	S	R
MZ4.1, MZ4.2, MZ4.3	Chiaramonte G.	Ragusa	R	R	R	R
2011						
DB1.7	Caltagirone	Catania	R	S	S	R
MA6.5, MA6.9	Mazzarrone	Catania	S	R	R	S

319 **Table 3**

MA7.2	Mazzarrone	Catania	S	R	R	R
LC3.6	Licodia Eubea	Catania	R	R	S	R
FG7.2	Chiaramonte G.	Ragusa	R	R	R	R
2012						
SP5.6, SP5.9, MA9.2	Mazzarrone	Catania	S	R	R	S
SV3.9	Licodia Eubea	Catania	S	R	R	R
MT6.4	Chiaramonte G.	Ragusa	S	R	R	S
DC3.9	Chiaramonte G	Ragusa	R	R	S	R
MT5.2	Chiaramonte G.	Ragusa	R	R	R	R
2013						
SR7.3	Licodia Eubea	Catania	R	S	S	R
NC4.12	Caltagirone	Catania	R	S	S	R
FN2.9	Mazzarrone	Catania	S	R	R	S
FN2.1	Mazzarrone	Catania	R	R	R	R
PT2.4, PT2.7, PT2.8	Chiaramonte G.	Ragusa	S	R	R	S
PD3.1, PD3.9	Chiaramonte G.	Ragusa	R	R	R	R

Botrytis cinerea isolates with multiple fungicide-resistance obtained from 'Mazzarrone grape PGI ' district.

[a] R and S indicate *in vitro* resistant and sensitive isolates, respectively.

3.3. Molecular data

Nucleotide sequences from isolates resistant or with reduced sensitivity to boscalid were compared with the corresponding nucleotide sequences of the sensitive isolates, with the reference wild-type sensitive strain (T4), and a complete SDH gene sequence (GenBank accession no. AY726618.1) was used for alignment. A single-nucleotide substitution in the *Sdh*B gene coding the Fe-S protein sub-unit (Ip) of succinate dehydrogenase was detected in 11/23 of boscalid-resistant isolates tested. In detail, 8 boscalid-HR (EC_{50} > 50 µg mL^{-1}) isolates showed a mutation at codon 272 with codon TAC instead of CAC. The nucleotide change from C to T led to the substitution of tyrosine with histidine (H272R) within the third cysteine-rich cluster-Ip sub-unit. The other 3 boscalid-R (EC_{50} between 20 and 50 µg mL^{-1}) isolates showed a mutation at codon 272 of CGC instead of CAC with the substitution of histidine with arginine (H272R). The

nucleotide sequences of *Sdh*B were identical in the boscalid-sensitive isolates and in the reference isolate (Fig. 3). No isolate was found to possess a mutation at codon 225, responsible for proline with leucine substitution. The remaining 12 isolates, found to be phenotypically resistant to boscalid (EC_{50} values within 5–19.99 μg mL^{-1}) in *in vitro* assays, showed no mutation in *Sdh*B.

Mutations in the nucleotide sequences were observed in all isolates showing *in vitro* resistance to carbendazim. In this case, the resistance was correlated with a point mutation at codon 198 in the β-tubulin gene in comparison with the reference sensitive isolate SAS56 (Fig. 3). At this codon, these isolates had the codon GCG rather than GAG, which resulted in the substitution of glutamic acid by alanine (*BenA* E198A). Molecular analysis of the sensitive isolates did not reveal any mutations in this β-tubulin gene fragment.

The well-known mutation (Banno et al., 2008) in the sequence of BcOS1 gene that confers resistance to dicarboximide iprodione was detected in 20 isolates at codon 365 (ATC→AGC - I365S), while a change in the remaining 11 isolates was detected at codon 369 (CAG→CCG - Q369P) encoding proline rather than glutamine, and codon 373 (AAC→AGC - N373S) encoding serine instead of asparagine (Fig. 3). Moreover, some isolates showing the first type mutation (at codon 365) also showed a mutation at codon 361, which was not significant because it encoded the same amino acid (glycine) (see black box in Fig. 3)

Fig. 3. Different mutations detected in partial nucleotide sequences for SdhB (at codon 272), β-tubulin (at codon 198), and BcOS1 (at codons 365, 369 and 373) genes respectively involved into boscalid, carbendazim and iprodione resistance in *Botrytis cinerea*.

3.4. Assays on grape berries

The data regarding fungicide sensitivity *in vivo* are reported in Table 4. Boscalid fungicide always provided a significant reduction (higher than 63%) of grey mould decay on grape berries caused by S isolates, whereas the lesion size reductions induced by R and HR *B. cinerea* isolates were not significant. The resulting percentages of sites infected by S isolates were significantly lower than those detected for R and HR pathogen isolates.

Similar data on fungicide efficacy were detected for both thiophanate-methyl and iprodione. Indeed, the percentages of sites fungicide-treated and infected by S isolates were always

369 significantly lower than those detected for R isolates of *B. cinerea*. Moreover, the reductions in
370 lesion size caused by S isolates on fungicide treated grape berries were significant, whereas
371 reductions were not significant for R isolates with the exception of iprodione against isolate
372 MZ4.2 (Table 4).

373 No lesions were observed on pyrimethanil-treated grape berries when S isolates of *B. cinerea*
374 were used for the inoculation. In contrast, pyrimethanil partially failed to control grey mould
375 decay caused by R isolates of *B. cinerea.* Indeed, these latter isolates were able to cause heavy
376 decays on fungicide treated berries (Table 4).

377 Fenhexamid and fludioxonil provided reductions of grey mould decay always higher than 87%
378 and 83%, respectively and no significant differences for percentages of infected sites were
379 detected among tested isolates (*data not shown*).

380

381 *3.5. Assays on grapevine leaves*

382

383 The R and HR boscalid isolates caused visible lesions on grapevine leaves previously treated
384 with the fungicide (Table 4). Indeed, these isolates produced lesions on fungicide-treated leaves
385 which did not significantly differ in diameter from those on control leaves. A low fungicide
386 efficacy in controlling grey mould decay (20.8–41.0% disease reduction) was detected in the RS
387 boscalid isolates. The greatest reductions in disease severity (63.1–100%) were detected in all S
388 isolates.

389 Regarding thiophanate-methyl and pyrimethanil, all isolates considered resistant in previous
390 assays infected fungicide-treated grapevine leaves, producing extensive lesions which were
391 comparable to those observed on untreated controls. No sensitive isolate caused severe symptoms
392 of decay on leaves (disease reduction of 59.6–95.7%).

393 Grapevine leaves treated with iprodione at label rate and then inoculated with sensitive isolates
394 were protected from infection (0.0% of infected sites on treated leaves), whereas those inoculated

with resistant isolates were not protected and showed heavy disease symptoms on leaves (66.7–100% of infected sites). However, for isolates MZ2.1 and MZ2.2, iprodione weakly reduced their development (44.7–72.4% disease reduction) and lesion diameters were significantly less than for controls; thus, these isolates were considered weakly resistant to iprodione.

Fenhexamid and fludioxonil markedly controlled infection caused by *B. cinerea* strains tested on grapevine leaves (disease reduction of 92.8–100%) and no significant differences were detected among tested isolates. The diameter of lesions on leaves treated with fungicides and subsequently inoculated with pathogen isolates were significantly lower than those of untreated leaves (*data not shown*).

Table 4

Infected sites (%) and lesion diameter (mm) on grape berries and grapevine leaves treated with different fungicides and inoculated with *Botrytis cinerea* isolates sensitive or resistant to active ingredients.

		Detached grape berries [b]				Grapevine leaves on seedlings [b]			
Fungicide	Isolates	Infected	Lesion (mm) [d]		Reduction	Infected	Lesion (mm) [d]		Reduction
Phenotype[a]		sites (%)[c]	Control	Treated	(%)	sites (%)[c]	Control	Treated	(%)
Boscalid									
S	BN5	66.7 a	25.5 *	7.8 *	69.4	55.6 b	20.6 *	7.6 *	63.1
S	CR6	56.7 a	12.2 *	4.5 *	63.1	0.0 a	9.7 *	0.0 *	100.0
RS	SR1	100.0 b	21.0 [ns]	24.8 [ns]	–	100.0 c	24.0 *	19.0 *	20.8
RS	SR5	100.0 b	28.6 [ns]	26.8 [ns]	6.3	100.0 c	25.1 *	14.8 *	41.0
R	MZ2.11	96.7 b	15.0 [ns]	16.6 [ns]	–	100.0 c	23.1 [ns]	22.8 [ns]	1.3
HR	MZ4.2	100.0 b	27.1 [ns]	25.7 [ns]	5.2	100.0 c	18.3 [ns]	17.6 [ns]	3.8
HR	MZ4.3	100.0 b	26.2 [ns]	19.5 [ns]	25.6	100.0 c	19.4 [ns]	16.0 [ns]	17.5
Iprodione									
S	CR5	30.0 a	8.3 *	2.9 *	65.1	0.0 a	10.0 *	0.0 *	100.0
S	DN1	50.0 b	20.0 *	5.7 *	71.5	0.0 a	23.2 *	0.0 *	100.0
R	MZ2.1	100.0 c	18.4 [ns]	20.1 [ns]	–	88.9 b	21.9 *	12.1 *	44.7
R	MZ2.2	100.0 c	21.7 [ns]	26.1 [ns]	–	66.7 b	21.0 *	5.8 *	72.4
R	MZ4.2	100.0 c	27.1 *	19.7 *	27.3	100.0 b	18.3 [ns]	15.7 [ns]	14.2
R	MZ4.3	100.0 c	26.2 [ns]	20.2 [ns]	22.9	100.0 b	19.4 *	15.7 *	19.1
Thiophanate-methyl									
S	MTK4	30.0 a	23.8 *	5.4 *	77.3	11.1 a	23.3 *	1.0 *	95.7
S	MTR6	33.3 a	23.6 *	3.6 *	84.7	11.1 a	20.3 *	1.0 *	95.1

	R	MZ2.1	100.0 b	18.4 [ns]	12.4 [ns]	32.6	100.0 b	21.9 [ns]	21.2 [ns]	3.2
	R	MZ2.2	100.0 b	18.1 [ns]	22.7 [ns]	–	100.0 b	21.0 [ns]	20.9 [ns]	0.5
	R	MZ2.11	93.3 b	13.6 [ns]	16.7 [ns]	–	100.0 b	23.1 [ns]	21.2 [ns]	8.2
	R	MZ4.2	100.0 b	27.1 [ns]	26.9 [ns]	0.7	100.0 b	18.3 [ns]	18.9 [ns]	–
	R	MZ4.3	100.0 b	26.2 [ns]	26.4 [ns]	–	100.0 b	19.4 [ns]	18.0 [ns]	7.2
Pyrimethanil										
	S	BN1	0.0 a	17.6 *	0.0 *	100.0	55.6 a	14.1 *	5.7 *	59.6
	S	MZ3.1	0.0 a	9.5 *	0.0 *	100.0	44.4 a	12.3 *	4.2 *	65.8
	R	FG4	53.3 b	11.7 [ns]	5.2 [ns]	55.5	100.0 b	22.0 [ns]	22.4 [ns]	–
	R	SR5	40.0 b	24.5 *	15.1 *	38.4	100.0 b	25.1 *	18.4 *	26.7
	R	MZ4.2	100.0 c	27.1 *	19.9 *	26.6	100.0 b	18.3 [ns]	18.7 [ns]	–
	R	MZ4.3	100.0 c	26.2 *	20.7 *	21.0	100.0 b	19.4 [ns]	15.8 [ns]	18.6

[a] S = sensitive isolate; RS = isolates with reduced sensitivity, and R = resistant isolates based on in vitro and molecular tests.

[b] Each data point represents the mean of 30 values (10 berries per 3 replicates) for detached grape berry assay and 18 (6 plugs per 3 leaves) for grapevine leaf assays respectively corresponding to the same number of wounded sites.

[c] Sites where infection starts have been percentage calculated only in fungicide-treated leaves after 6 and 4 days for grape berries and grapevine leaves, respectively. These data were compared within each column among examined isolates according to Fisher's least significance difference test ($P = 0.01$).

[d] Mean data followed by *, within each row between control and treated leaves, denote significant differences at $P < 0.01$ according to Mann Whitney non parametric rank test ($z > 2.58$); ns: not significant.

4. Discussion

This paper provides first data on resistance and/or sensitivity of *B. cinerea* isolates collected from main table grape production in Sicily to six fungicides belonging to chemical groups with different modes of action.

Overall, this study documents the field occurrence *B. cinerea* isolates with multiple resistance to different botryticides (benzimidazoles, dicarboximides, anilinopyrimidines and SDHIs). Multiple fungicide resistance of grey mould was previously reported in German, Chilean, and Italian (Piedmont and Apulia) vineyards (De Miccolis Angelini et al., 2014; Gullino et al., 2000; Latorre and Torres, 2012; Leroch et al., 2011) and in other crops worldwide (Bardas et al., 2010; Fernández-Ortuño et al., 2014; Moyano et al., 2004; Myresiotis et al., 2007; Sun et al., 2010). Isolates resistant to both old and new botryticides have emerged over time in many crops

429 worldwide (Amiri et al., 2014; Grabke et al., 2013; Leroux, 2007; Saito et al., 2014; Yin et al.,
430 2014). However, the resistant isolates detected in some studies have only been characterized
431 phenotypically.

432 Fungicide resistance of *B. cinerea* isolates, detected in our *in vitro* assays, was confirmed by
433 breakdown in efficacy detected in *in vivo* experiments. Additionally, molecular analysis has
434 revealed point mutations directly involved in the nucleotide sequences of β-tubulin, *Sdh*B and
435 BcOS1 histidine kinase genes that conferred resistance to carbendazim, boscalid (SDHI) and
436 iprodione (dicarboximide), respectively.

437 Currently, field resistant isolates of *B. cinerea* to boscalid have been reported in a limited
438 number of hosts (Amiri et al., 2014; Bardas et al., 2010; Fernández-Ortuño et al., 2014; Veloukas
439 et al., 2011; Yin et al., 2011) including grape in Germany (Wine Road region), France
440 (Champagne region) and, more recently, in Italy (Apulia region) (De Miccolis Angelini et al.,
441 2014; Leroch et al., 2011; Leroux et al., 2010). The low frequency of boscalid-resistant genotypes
442 of *B. cinerea* detected in Sicilian vineyards and conferred by the *Sdh*B$^{H272R/Y}$ mutation, could be
443 due both to its relatively recent introduction (2006 in Italy) and after the product launch farmers
444 did not use the fungicide frequently, performing a maximum of one application per growing
445 season in recent years. Boscalid–R isolates were detected from all municipalities within the
446 Catania province (Licodia Eubea, Caltagirone and Mazzarrone) although with a very low number
447 per municipality, whereas boscalid-R isolates were collected exclusively in one municipality in
448 Ragusa (i.e. Chiaramonte Gulfi), which incidentally is the most representative for typical grape
449 production in this province. This suggests that the fungicide may yet be included in integrated
450 management programs for control of botrytis bunch rot of 'Mazzarrone grape PGI'. However, the
451 field application of this botryticide should be approached with caution since some pathogen
452 isolates possessed boscalid-resistance while other isolates showed an *in vitro* and *in vivo*
453 decreased sensitivity to the fungicide.

454 The frequency of benzimidazole-resistant genotypes of *B. cinerea* was found to be relatively
455 low in the detected area and it was associated with the most common worldwide E198V mutation
456 in the β-tubulin gene as reported in other papers (Banno et al., 2008; Ma and Michailides, 2005).
457 This could be partially explained by no or irrelevant use of benzimidazoles in the last decade and,
458 therefore, the almost lack of selection pressure exerted by the fungicide may have induced an
459 increase in wild type (sensitive) isolates having a higher fitness and, consequently, higher
460 competitive activity than resistant isolates. However, the latter isolates could persist within
461 population for a long time also in absence of benzimidazole applications (Brent and Hollomon,
462 2007a).
463 Regarding the dicarboximides, few isolates exhibited resistance to iprodione, showing both the
464 well-known point mutation (type I) at amino acid position 365 (I365S) and amino acid
465 substitutions of type III at position 369 (Q369P) and 373 (N373S) in the histidine kinase genes
466 (*BcOS1*) (Banno et al., 2008). The most dicarboximides-resistant isolates also showed resistance
467 to benzimidazoles, confirming previous data that reported this double resistance in *B. cinerea*
468 populations occurring in a variety of crops (Beever et al., 1989; Brent and Hollomon, 2007a;
469 Yourman and Jeffers, 1999).
470 The high frequency of pyrimethanil-resistant isolates detected in this survey could be related
471 to the widespread use of this fungicide. Resistance to pyrimethanil has developed worldwide and
472 a high percentage of anilinopyrimidine-resistant isolates has been reported in Italy, France,
473 Switzerland, Greece, China and Australia, suggesting that there is a high risk for the occurrence
474 of anilinopyrimidine resistance in *B. cinerea* populations (Baroffio et al., 2003; Chapeland et al.,
475 1999; Gullino et al., 2000; Latorre et al., 2002; Leroux et al., 1999; Myresiotis et al., 2007;
476 Sergeeva et al., 2002; Sun et al., 2010).
477 Regarding fenhexamid and fludioxonil, no fungicide-resistant field isolate was found within
478 our *B. cinerea* population although these compounds have been widely used in Sicilian vineyards.
479 These findings contrast with the data on reduced sensitivity of *B. cinerea* field strains to

fenhexamid detected in Chilean, French and Swiss vineyards (Baroffio et al., 2003; Esterio et al., 2007; Billard et al., 2012) and on other crops worldwide (Myresiotis et al., 2007; Leroux, 2007; Ma and Michailides, 2005). Thus, this molecule is classified as a low risk for the resistance development by FRAC (Brent and Hollomon, 2007b; FRAC Code List) and its use for controlling of grey mould of grape should be encouraged since it also shows a low persistence in the environment (Abbate et al., 2007) On the contrary, for fludioxonil, our data are in accordance with previous reports worldwide in several hosts, where the occurrence of fludioxonil resistance was not observed, or rarely observed, in *B. cinerea* populations (Baroffio et al., 2003; De Miccolis Angelini et al., 2014; Ferñandez-Ortuño et al., 2013; Grabke et al., 2014; Latorre and Torres, 2012; Leroch et al., 2012; Yin et al., 2014; Zhao et al., 2010). Some of these resistant isolates could have fitness penalties (Zhao et al., 2010), which may at least partly explain the absence and/or low frequency of fungicide-resistant isolates within fungal populations in the field detected here and in other studies (Ferñandez-Ortuño et al., 2013; Leroch et al., 2012). Comparative data regarding sensitivity/resistance of *Botrytis cinerea* to fluodioxonil and iprodione confirmed past study, according to which dicarboximide-resistant field isolates proved to be sensitive to fludioxonil, but the latter did not select for dicarboximide resistance in field experiments (Hilber *et al*., 1994, Brent and Hollomon, 2007a).

This finding indicates that fenhexamid and fludioxonil also have great potential for control of grey mould on table grape in the PGI 'Mazzarrone grape' district.

Our isolates showing multiple fungicide resistance displayed a considerable ability to infect grape berries and leaves pre-treated with the tested fungicides at their label rates. Therefore, a shift towards reduced sensitivity in *B. cinerea* to the above-mentioned compounds could be predictive of the breakdown of fungicide efficacy for this important table grape area production. The detection of *B. cinerea* isolates with multiple resistance to these botryticides in the field, although with low frequency, actually could represent a serious threat for typical 'Mazzarrone grape PGI ' since the pathogen is classified at 'high risk' for resistance development (EPPO, 2002;

506 Russel, 2004) – due to its polycyclic nature, abundant inoculum production, efficient
507 dissemination mechanisms and wide host range (Myresiotis et al., 2007). Recently, Kretschmer et
508 al. (2009) showed that the mechanism of multiple fungicide resistance for plant pathogens could
509 be additionally due to decreased accumulation of compounds in the mycelium caused by
510 increased fungicide efflux.

511 An effective anti-resistance strategy can best be achieved by preventing large-scale field
512 resistance in vineyards and cannot rely on a single or few fungicides. In light of these findings,
513 the use of benzimidazoles, dicarboximides, anilinopyrimidines and the SDHI boscalid within
514 Sicilian districts should be performed in alternation or in mixtures with botryticides having
515 different modes of action and showing a low risk of resistance development such as
516 phenylpyrroles and hydroxyanilides. The results of the present study indicate that, by continuous
517 selection of multi-resistant isolates, chemical control of grey mould in vineyards will become
518 increasingly difficult in this important Italian area of table grape production. Thus, careful
519 monitoring of sensitivity and multiple resistance among botryticides over time will be crucial
520 point in managing fungicide resistance.

521

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683
684 **Figure Captions**

685

686 **Fig. 1.** Frequency distribution of EC_{50} values for boscalid, carbendazim, iprodione, pyrimethanil,
687 fenhexamid and fludioxonil among 302 isolates of *Botrytis cinerea* collected from different
688 vineyards in Sicily.

689 **Fig. 2.** Venn diagram indicating simultaneous resistance to boscalid, carbendazim, iprodione and
690 pyrimethanil among 302 *Botrytis cinerea* isolates collected from table grape vineyards during
691 2009-2013. EC_{50} values higher than 1 µg ml^{-1} (carbendazim, iprodione and pyrimethanil) and 5
692 µg ml^{-1} (boscalid) classified isolates as resistant and/or with reduced sensitivity to fungicides.
693 The large circle represents the full set of 302 isolates tested for fungicide sensitivity. Each of four
694 smaller circles represents the set of isolates with reduced sensitivity to the corresponding active
695 ingredients. The intersections among different circles indicates 4 subgroups that were
696 simultaneously resistant to more than one fungicide.

697 **Fig. 3.** Different mutations detected in partial nucleotide sequences for SdhB (at codon 272), β-
698 tubulin (at codon 198), and BcOS1 (at codons 365, 369 and 373) genes respectively involved into
699 boscalid, carbendazim and iprodione resistance in *Botrytis cinerea*.