

Scotland's Rural College

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

3

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9

10 **ABSTRACT**

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

28

29 *Keywords:*

30 *Botrytis cinerea*

31 multiple fungicide resistance

32 table grape

33 boscalid

34

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38

## 39 **1. Introduction**

40

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

58 mutation in the genes encoding the target site protein causing reduced fungicide binding. These  
59 modifications, often determining the 'specific resistance' towards a single or one class of  
60 fungicide, were first detected for anti-microtubule fungicides (e.g. MBCs), and successively  
61 verified for dicarboximides, hydroxyanilides, strobilurins, and SDHIs (Fillinger et al., 2008;  
62 Leroux et al., 2002, 2010). Besides specific resistances, multiple fungicide resistance has also  
63 been recently detected in French and German vineyards, but it usually exhibits considerable  
64 resistance levels towards several classes of botryticides that are mediated by a single gene  
65 (Kretschmer et al., 2009). In the past, fungicide resistance within some *B. cinerea* populations  
66 was reported on several crops (Amiri et al., 2013; Baroffio et al., 2003; Brent and Hollomon,  
67 2007a; Myresiotis et al., 2007; Weber, 2011). Field resistance of *B. cinerea* to various fungicides  
68 has also been detected in vineyards worldwide, resulting in poor fungicide efficacy (Beever et al.,  
69 1989; Latorre et al., 2002; Latorre and Torres, 2012; Leroux, 2007; Sergeeva et al., 2002). The  
70 use of site-specific fungicides to control high resistance risk pathogens, such as *B. cinerea*, may  
71 further increase the development of field resistance (Brent and Hollomon, 2007b). Therefore,  
72 continuous monitoring of fungicide resistance is crucial following the first detection of resistant  
73 genotypes in vineyards to ensure that adequate anti-resistance strategies are implemented to  
74 prevent or delay breakdown of fungicide efficacy.

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

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## 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<sup>®</sup> [active ingredient (a.i.) pyrimethanil] and Switch<sup>®</sup> (a.i. cyprodinil + fludioxonil) (up to two applications per season), Cantus<sup>®</sup> (a.i. boscalid) and Teldor Plus<sup>®</sup> (a.i. fenhexamid) (one application per season). Thiophanate-methyl (Enovit Metil<sup>®</sup>) and iprodione (Rovral Plus<sup>®</sup>) 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

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

117

118 **Table 1**

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

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

121 <sup>a</sup> Used in *in vitro* assays. Bavistin is not registered for the use on grape.

122 <sup>b</sup> Used in bioassays.

123 °WG, water dispersible granule; SC, suspension concentrate.

124

### 125 2.3. Fungicide sensitivity

126

127 The sensitivity of *B. cinerea* isolates to fungicides was assessed by measuring radial growth on  
128 agar plates amended with different concentrations of a.i.(s). All fungicides were tested on PDA  
129 except for pyrimethanil and boscalid, which were tested on a minimal medium containing 10 g of  
130 glucose, 1.5 g of K<sub>2</sub>HPO<sub>4</sub>, 2 g of KH<sub>2</sub>PO<sub>4</sub>, 1 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 g yeast  
131 extract and 12.5 g of agar (Oxoid) per liter of distilled H<sub>2</sub>O (Hu et al., 2011; Myresiotis et al.,  
132 2007, 2008). Yeast extract was not added in the sensitivity assay for pyrimethanil (Myresiotis et  
133 al., 2007). Autoclaved agar media were cooled to about 45°C and amended with appropriate  
134 volumes of the fungicide stock solutions to obtain the following a.i. concentrations: 0.05, 0.5, 1,  
135 5, 10, 20 and 50 µg mL<sup>-1</sup> for boscalid; 0.01, 0.1, 1, 10 and 100 µg mL<sup>-1</sup> for carbendazim; 0.001,  
136 0.005, 0.01, 0.05, 0.1 and 1 µg mL<sup>-1</sup> for fenhexamid and fludioxonil; 0.1, 1, 5, 10 and 20 µg mL<sup>-1</sup>  
137 for iprodione and 0.01, 0.05, 0.1, 1, 5, 10 and 50 µg mL<sup>-1</sup> for pyrimethanil. Unamended media  
138 plates served as controls. Mycelium plugs, cut from the edge of an actively growing culture on  
139 agar media, were placed upside down on the centre of each fungicide-amended or control dish.  
140 Dishes were incubated at 20 °C in darkness for 3–5 days. For each concentration, three plates  
141 were used and colony diameter was measured in two perpendicular directions, subtracting the  
142 original diameter of the mycelium plug (6 mm) for the calculated value. These assays were  
143 performed twice. Radial growth on each plate was measured and the raw data from three  
144 replicates used to calculate growth reduction (GR) = [1 – (radius in amended plates/radius of  
145 control plates)] × 100. The effective fungicide concentration to inhibit 50% of mycelial growth  
146 (EC<sub>50</sub>) was calculated for each isolate by linear regressions of the mycelial growth reductions  
147 versus the log<sub>10</sub> transformation of the fungicide concentrations. Frequency distributions of the  
148 isolates between the intervals of EC<sub>50</sub> values were established.

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

157

#### 158 2.4. Molecular analysis

159

160 To identify the mutations correlated with resistance to boscalid, the complete coding sequence  
161 of the *sdhB* subunit (complete succinate dehydrogenase iron sulphur protein gene) of  
162 representative *B. cinerea* isolates, selected on the basis of phenotypic sensitivity to the fungicide  
163 (sensitive or resistant) in *in vitro* assays, was compared to the corresponding gene sequence of the  
164 reference sensitive strain T4 of *Botryotinia fuckeliana* (GenBank accession no. AY726618.1).  
165 The resistance to the MBC "carbendazim" was identified by comparing the coding sequences of  
166  $\beta$ -tubulin of the tested *B. cinerea* strains to the corresponding gene sequence of the reference  
167 sensitive strain SAS56 (GenBank accession no. Z69263.2). The same approach was also used to  
168 identify mutations correlated to resistance to iprodione; here, the coding sequences of *BcOSI*  
169 genes (coding for histidine kinase) of the *B. cinerea* strains were compared to reference sensitive  
170 strain Bc56 (GenBank accession no. AB064962.1). Genomic DNA was extracted and purified  
171 from mycelia of *B. cinerea* isolates grown on PDA for 5 days in darkness. Mycelia were  
172 harvested and washed in SDW, frozen in liquid nitrogen and lyophilized. DNA from each isolate  
173 was extracted using the kit Wizard<sup>®</sup> Magnetic DNA Purification System for Food (Promega,  
174 Madison, USA). The purified DNA was eluted in a final volume of 100  $\mu\text{L}$  and checked by



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

196

## 197 2.5. Assays on grape berries

198

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

201 *submitted*). At least two sensitive and four to five resistant isolates or isolates with reduced  
202 sensitivity to each fungicide were selected according to both *in vitro* and molecular data. Single  
203 detached berries with pedicel were surface disinfected with 2% of NaOCl for 2 min and rinsed  
204 twice in SDW. After drying, four wounds (1-2 mm deep) were made with a sterile hypodermic  
205 needle before being sprayed with a fungicide suspension. Boscalid, fenhexamid and fludioxonil  
206 a.i.(s) were used at 500 mg L<sup>-1</sup>, iprodione at 750 mg L<sup>-1</sup>, pyrimethanil at 800 mg L<sup>-1</sup>, and  
207 thiophanate-methyl at 1 g L<sup>-1</sup>, respectively. These dosages reflect the rates recommended for  
208 botrytis bunch rot of table grape for six commercial formulations registered in Italy (Table 1).  
209 Thirty berries were used for each treatment (10 berries/replicate) and placed in a cage containing  
210 an aluminum tray at the bottom of which a thin layer of water was poured to maintain high  
211 relative humidity (RH). Treatments were applied with a hand-pump until berries were thoroughly  
212 wet. After 6 h, the berries were inoculated by placing a 20 µL drop of the conidial suspension (1-  
213  $2 \times 10^5$  conidia mL<sup>-1</sup>) obtained by flooding 10 day-old sporulating cultures on PDA plates with  
214 SDW at the surface of the wounds. Berries were placed in separate rows (40 mm apart) on  
215 expanded metal sheets in clear plastic-covered cages. The same number of berries sprayed with  
216 SDW served as control. For each isolate, lesion diameter (severity of decay) on each berry and  
217 the number of infected berries per treatment (disease incidence) were recovered after 6 days of  
218 incubation at 24–25 °C. Severity of grey mould decay was calculated both on treated and control  
219 grape berries determining its relative reduction of botrytis rot (control efficacy %). The  
220 experiment was performed twice.

221

## 222 2.6. Assays on grapevine leaves

223

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

227 70% RH with a photoperiod of 16 h. Subsequently, the plants were sprayed to run-off with the  
228 fungicide suspensions at the same rates used in the previous assay. After two hours, the leaves of  
229 these plants were inoculated with selected *B. cinerea* isolates. Six mycelial plugs removed from  
230 the margin of the colonies growing on PDA were placed on the upper surface of each leaf. Three  
231 leaves (i.e. three replicates) were used for each isolate. The control plants were sprayed with  
232 SDW and then inoculated with PDA plugs containing *B. cinerea* mycelium. To create favorable  
233 conditions for infection, inoculated plants were covered with plastic bags and incubated in the  
234 growth chamber at 25 °C with a photoperiod of 16 h and high RH (90–95%). The disease  
235 incidence and diameters of the developing lesions were measured 4 days after inoculation.  
236 Severity of grey mould infections was compared between treated and control grape leaves and  
237 relative reductions were determined for each isolate. The experiment was carried out twice.

238

## 239 2.7. Data analysis

240

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

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

252

### 253 3. Results

254

#### 255 3.1. Pathogen sensitivity to fungicides

256

257 The EC<sub>50</sub> 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<sub>50</sub> values to  
259 boscalid. Among them, 254 (84.1%) were classified as highly sensitive to boscalid (HS), since  
260 their EC<sub>50</sub> < 1 µg mL<sup>-1</sup>, whereas 25 isolates (8.3%) had EC<sub>50</sub> values between 1 and 4.99 µg mL<sup>-1</sup>  
261 and were considered as sensitive (S) isolates. The values for most of these isolates fell within  
262 0.1–0.49 µg mL<sup>-1</sup> range (Fig. 1–A). The remaining 23 isolates (7.6%) grew on media  
263 supplemented with boscalid concentrations of 5 µg mL<sup>-1</sup> or more (Table 2). In detail, 12 isolates  
264 (4%) had EC<sub>50</sub> values ranging from 5 to 19.99 µg mL<sup>-1</sup> (RF values within 5–20 range) and were  
265 considered as reduced sensitivity (RS) phenotypes, three (1%) had EC<sub>50</sub> between 20 and 49.99  
266 µg mL<sup>-1</sup> and eight (2.6%) isolates had EC<sub>50</sub> values higher than 50 µg mL<sup>-1</sup> (Fig. 1–A). Isolates  
267 with EC<sub>50</sub> falling within the 20–50 µg mL<sup>-1</sup> range and having EC<sub>50</sub> > 50 µg mL<sup>-1</sup> 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<sub>50</sub> values  
270 less than 1 µg mL<sup>-1</sup> (Table 2). The remaining 42 isolates (13.9%), having EC<sub>50</sub> higher than 100  
271 µg mL<sup>-1</sup>, 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<sub>50</sub> values for these isolates ranged from 0.1 to  
274 0.69 µg mL<sup>-1</sup> with the highest frequency of values falling within 0.2–0.29 µg mL<sup>-1</sup>. Otherwise,  
275 31 isolates (10.3%) showed resistance to iprodione and grew on media amended with fungicide  
276 concentrations higher than 1 µg mL<sup>-1</sup> (Table 2, Fig. 1–C).

277 About 69.2% of the isolates were found sensitive to pyrimethanil (Fig. 1–D), with EC<sub>50</sub> values  
278 between 0.03 and 0.86 µg mL<sup>-1</sup>. For this fungicide, a high frequency of resistant isolates (30.8%)

279 was detected within the *B. cinerea* population since they grew on media amended with  
 280 pyrimethanil at concentrations higher than 1  $\mu\text{g mL}^{-1}$  (Table 2). Overall, 15.2% of isolates  
 281 exhibited an  $\text{EC}_{50}$  value within the 1.0–1.99  $\mu\text{g mL}^{-1}$  range, 7.0% showed  $\text{EC}_{50}$  values between  
 282 2.0 and 4.99  $\mu\text{g mL}^{-1}$  and 8.6% had an  $\text{EC}_{50}$  value higher than 5  $\mu\text{g mL}^{-1}$  (Fig. 1–D).

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

286

287 **Table 2**

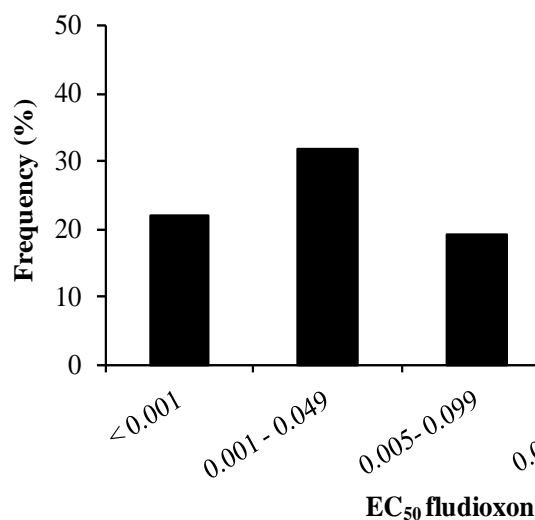
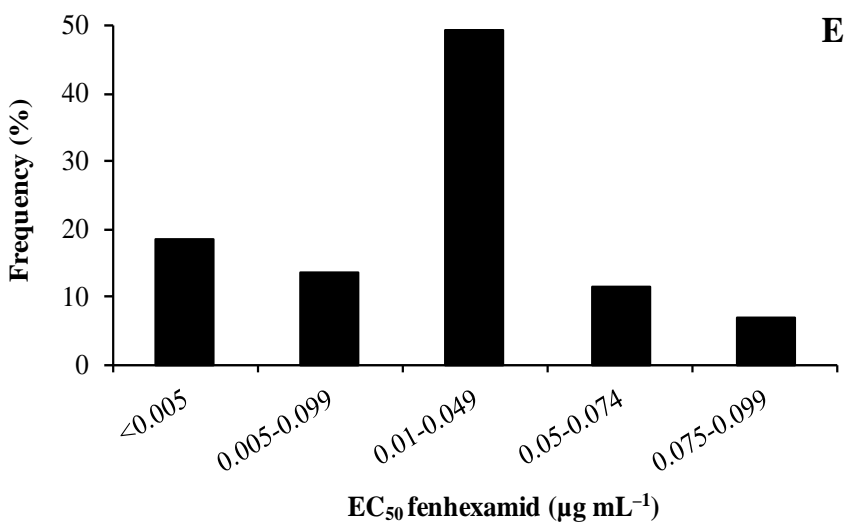
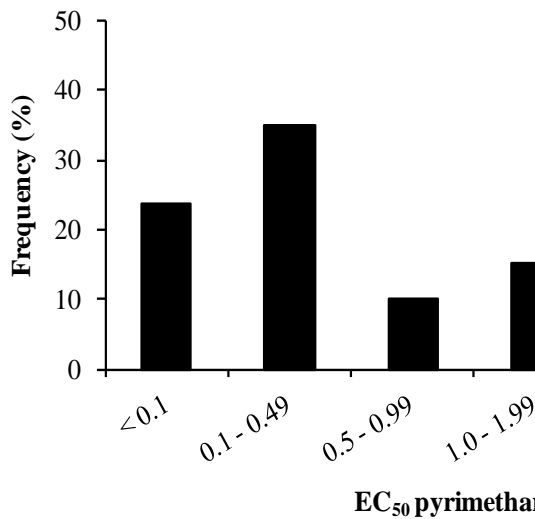
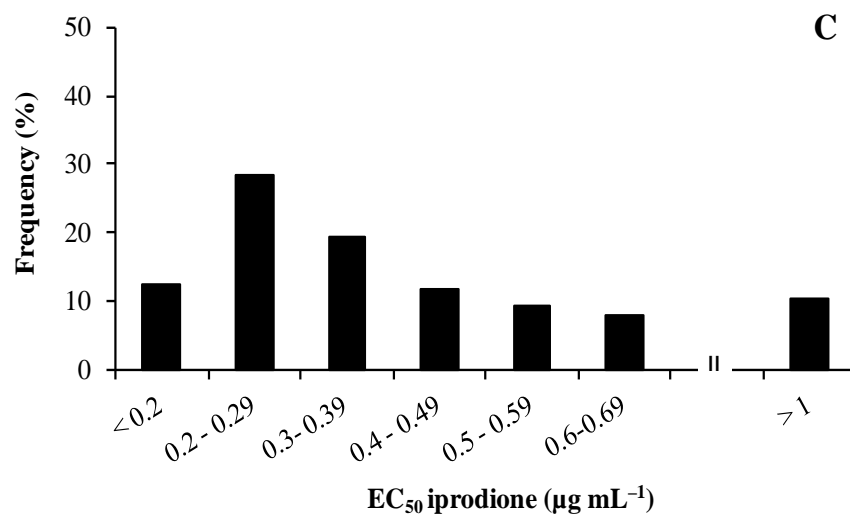
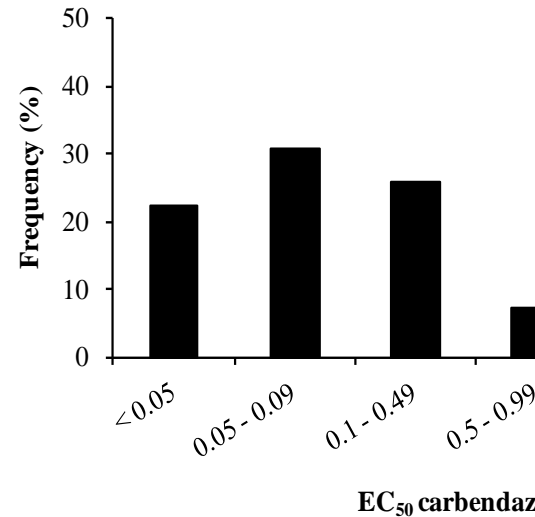
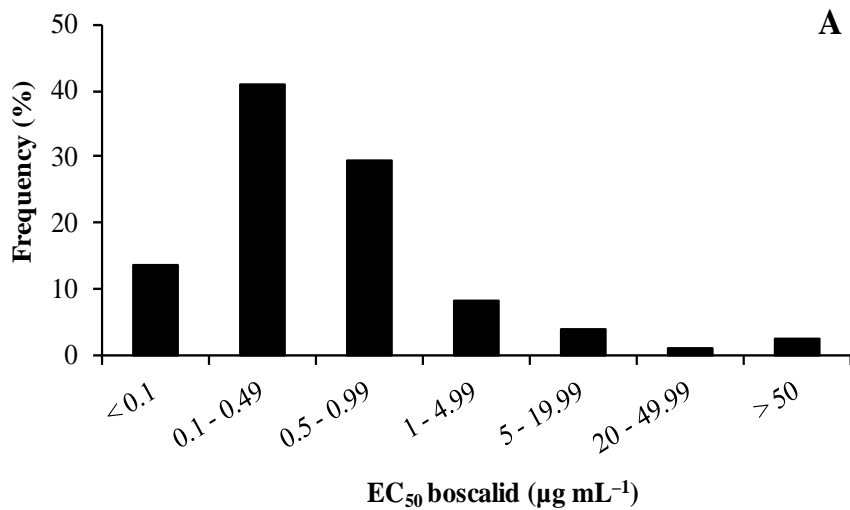
288 Sensitivity of *Botrytis cinerea* isolates from table grape to different tested fungicides.

Fungicide	$\text{EC}_{50}$ ( $\mu\text{g mL}^{-1}$ )		No. of isolates		Resistance frequency (%) <sup>a</sup>
	Sensitive	Resistant	Sensitive	Resistant	
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

289 <sup>a</sup> Resistance frequency values were determined based on discriminatory concentrations of 0.1  $\mu\text{g mL}^{-1}$  for  
 290 fenhexamid and fludioxonil, and 1  $\mu\text{g mL}^{-1}$  for boscalid, carbendazim, iprodione and pyrimethanil.

291

292



293

294

295

296

**Fig. 1.** Frequency distribution of EC<sub>50</sub> values for boscalid, carbendazim, iprodione, pyrimethanil, fenhexamid and fludioxonil among 302 isolates of *Botrytis cinerea* collected from different vineyards in Sicily.

297

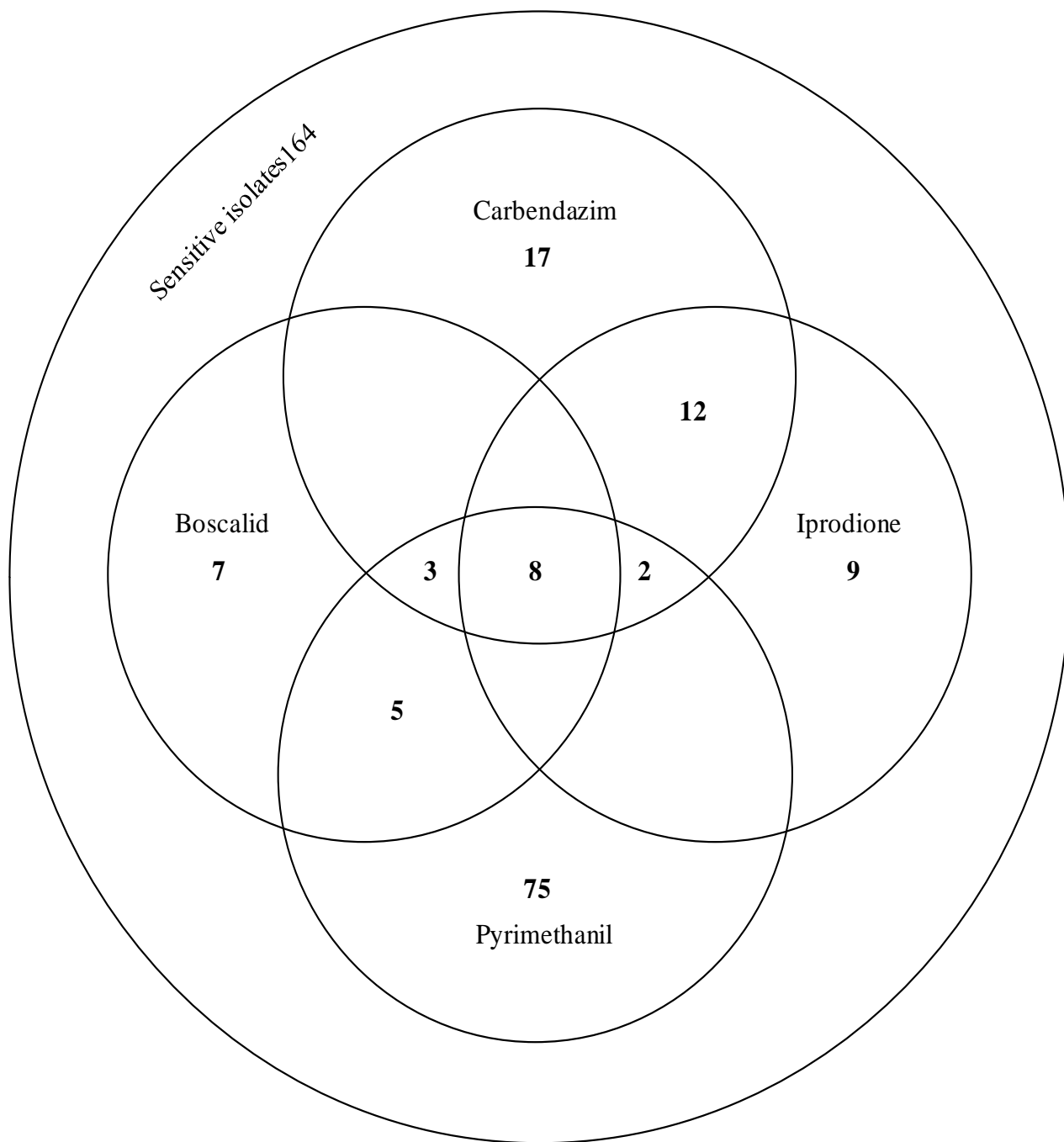
298 3.2. *Multiple resistance among fungicides*

299

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<sub>50</sub> values higher than 1 µg mL<sup>-1</sup> (carbendazim, iprodione and pyrimethanil) and 5 µg mL<sup>-1</sup> (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 <sup>a</sup>	S <sup>a</sup>	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

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

322 <sup>a</sup> R and S indicate *in vitro* resistant and sensitive isolates, respectively.

323

### 324 3.3. Molecular data

325

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

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

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

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

353

Fungicide sensitivity	Gene	Mutation type
	<i>SdhB</i>	
Boscalid-S		GATAACAGCATGAGTTTGTACAGATGTCACACTATTCTCAACTGCTCGAGG
<b>Boscalid-R (1)</b>		GATAACAGCATGAGTTTGTACAGATGT <b>TAC</b> ACTATTCTCAACTGCTCGAGG
<b>Boscalid-R (2)</b>		GATAACAGCATGAGTTTGTACAGATGT <b>CGC</b> ACTATTCTCAACTGCTCGAGG
Reference-S		GATAACAGCATGAGTTTGTACAGATGTCACACTATTCTCAACTGCTCGAGG
	<i>β-tubulin</i>	
Carbendazim-S		CTCTCTCTGTCCATCAATTGGTTGAGAACTCTGACGAGACCTTCTGTATCG
<b>Carbendazim-R (1)</b>		CTCTCTCTGTCCATCAATTGGTTGAGAACTCTGAC <b>GCG</b> ACCTTCTGTATCG
Reference-S		CTCTCTCTGTCCATCAATTGGTTGAGAACTCTGACGAGACCTTCTGTATCG
	<i>BcOS1</i>	
Iprodione-S		TCTTGGGGGTCAAGCAGAAATCGAAGGCGTCCAGGGCATGTGGAA CACATT
<b>Iprodione-R (1)</b>		TCTTGGG <b>GGC</b> CAAGCAGAA <b>AGC</b> GAAGGCGTCCAGGGCATGTGGAA CACATT
<b>Iprodione-R (2)</b>		TCTTGGGGGTCAAGCAGAAATCGAAGGCGT <b>CCG</b> GGCATGTGG <b>AGC</b> ACATT
Reference-S		TCTTGGGGGTCAAGCAGAAATCGAAGGCGTCCAGGGCATGTGGAA CACATT

354

355

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

359

### 360 3.4. Assays on grape berries

361

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

367 Similar data on fungicide efficacy were detected for both thiophanate-methyl and iprodione.

368 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

395 with resistant isolates were not protected and showed heavy disease symptoms on leaves (66.7–  
 396 100% of infected sites). However, for isolates MZ2.1 and MZ2.2, iprodione weakly reduced their  
 397 development (44.7–72.4% disease reduction) and lesion diameters were significantly less than for  
 398 controls; thus, these isolates were considered weakly resistant to iprodione.

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

404

405 **Table 4**

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

Fungicide Phenotype <sup>a</sup>	Isolates	Detached grape berries <sup>b</sup>				Grapevine leaves on seedlings <sup>b</sup>				
		Infected sites (%) <sup>c</sup>	Lesion (mm) <sup>d</sup>		Reduction (%)	Infected sites (%) <sup>c</sup>	Lesion (mm) <sup>d</sup>		Reduction (%)	
			Control	Treated			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 <sup>ns</sup>	24.8 <sup>ns</sup>	–	100.0 c	24.0 *	19.0 *	20.8	
RS	SR5	100.0 b	28.6 <sup>ns</sup>	26.8 <sup>ns</sup>	6.3	100.0 c	25.1 *	14.8 *	41.0	
R	MZ2.1	96.7 b	15.0 <sup>ns</sup>	16.6 <sup>ns</sup>	–	100.0 c	23.1 <sup>ns</sup>	22.8 <sup>ns</sup>	1.3	
HR	MZ4.2	100.0 b	27.1 <sup>ns</sup>	25.7 <sup>ns</sup>	5.2	100.0 c	18.3 <sup>ns</sup>	17.6 <sup>ns</sup>	3.8	
HR	MZ4.3	100.0 b	26.2 <sup>ns</sup>	19.5 <sup>ns</sup>	25.6	100.0 c	19.4 <sup>ns</sup>	16.0 <sup>ns</sup>	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 <sup>ns</sup>	20.1 <sup>ns</sup>	–	88.9 b	21.9 *	12.1 *	44.7	
R	MZ2.2	100.0 c	21.7 <sup>ns</sup>	26.1 <sup>ns</sup>	–	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 <sup>ns</sup>	15.7 <sup>ns</sup>	14.2	
R	MZ4.3	100.0 c	26.2 <sup>ns</sup>	20.2 <sup>ns</sup>	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 <sup>ns</sup>	12.4 <sup>ns</sup>	32.6	100.0 b	21.9 <sup>ns</sup>	21.2 <sup>ns</sup>	3.2
	R	MZ2.2	100.0 b	18.1 <sup>ns</sup>	22.7 <sup>ns</sup>	–	100.0 b	21.0 <sup>ns</sup>	20.9 <sup>ns</sup>	0.5
	R	MZ2.11	93.3 b	13.6 <sup>ns</sup>	16.7 <sup>ns</sup>	–	100.0 b	23.1 <sup>ns</sup>	21.2 <sup>ns</sup>	8.2
	R	MZ4.2	100.0 b	27.1 <sup>ns</sup>	26.9 <sup>ns</sup>	0.7	100.0 b	18.3 <sup>ns</sup>	18.9 <sup>ns</sup>	–
	R	MZ4.3	100.0 b	26.2 <sup>ns</sup>	26.4 <sup>ns</sup>	–	100.0 b	19.4 <sup>ns</sup>	18.0 <sup>ns</sup>	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 <sup>ns</sup>	5.2 <sup>ns</sup>	55.5	100.0 b	22.0 <sup>ns</sup>	22.4 <sup>ns</sup>	–
	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 <sup>ns</sup>	18.7 <sup>ns</sup>	–
	R	MZ4.3	100.0 c	26.2 *	20.7 *	21.0	100.0 b	19.4 <sup>ns</sup>	15.8 <sup>ns</sup>	18.6

408 <sup>a</sup> S = sensitive isolate; RS = isolates with reduced sensitivity, and R = resistant isolates based on in vitro and molecular tests.

409 <sup>b</sup> Each data point represents the mean of 30 values (10 berries per 3 replicates) for detached grape berry assay and 18 (6 plugs per  
410 3 leaves) for grapevine leaf assays respectively corresponding to the same number of wounded sites.

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

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

416

#### 417 4. Discussion

418

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

422 Overall, this study documents the field occurrence *B. cinerea* isolates with multiple resistance  
423 to different botryticides (benzimidazoles, dicarboximides, anilinopyrimidines and SDHIs).  
424 Multiple fungicide resistance of grey mould was previously reported in German, Chilean, and  
425 Italian (Piedmont and Apulia) vineyards (De Miccolis Angelini et al., 2014; Gullino et al., 2000;  
426 Latorre and Torres, 2012; Leroch et al., 2011) and in other crops worldwide (Bardas et al., 2010;  
427 Fernández-Ortuño et al., 2014; Moyano et al., 2004; Myresiotis et al., 2007; Sun et al., 2010).  
428 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  $\beta$ -tubulin, *SdhB* 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 *SdhB*<sup>H272R/Y</sup> 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  $\beta$ -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 (*BcOSI*) (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

480 fenhexamid detected in Chilean, French and Swiss vineyards (Baroffio et al., 2003; Esterio et al.,  
481 2007; Billard et al., 2012) and on other crops worldwide (Myresiotis et al., 2007; Leroux, 2007;  
482 Ma and Michailides, 2005). Thus, this molecule is classified as a low risk for the resistance  
483 development by FRAC (Brent and Hollomon, 2007b; FRAC Code List) and its use for  
484 controlling of grey mould of grape should be encouraged since it also shows a low persistence in  
485 the environment (Abbate et al., 2007) On the contrary, for fludioxonil, our data are in accordance  
486 with previous reports worldwide in several hosts, where the occurrence of fludioxonil resistance  
487 was not observed, or rarely observed, in *B. cinerea* populations (Baroffio et al., 2003; De  
488 Miccolis Angelini et al., 2014; Fernández-Ortuño et al., 2013; Grabke et al., 2014; Latorre and  
489 Torres, 2012; Leroch et al., 2012; Yin et al., 2014; Zhao et al., 2010). Some of these resistant  
490 isolates could have fitness penalties (Zhao et al., 2010), which may at least partly explain the  
491 absence and/or low frequency of fungicide-resistant isolates within fungal populations in the field  
492 detected here and in other studies (Fernández-Ortuño et al., 2013; Leroch et al., 2012).  
493 Comparative data regarding sensitivity/resistance of *Botrytis cinerea* to fludioxonil and  
494 iprodione confirmed past study, according to which dicarboximide-resistant field isolates proved  
495 to be sensitive to fludioxonil, but the latter did not select for dicarboximide resistance in field  
496 experiments (Hilber *et al.*, 1994, Brent and Hollomon, 2007a).

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

499 Our isolates showing multiple fungicide resistance displayed a considerable ability to infect  
500 grape berries and leaves pre-treated with the tested fungicides at their label rates. Therefore, a  
501 shift towards reduced sensitivity in *B. cinerea* to the above-mentioned compounds could be  
502 predictive of the breakdown of fungicide efficacy for this important table grape area production.  
503 The detection of *B. cinerea* isolates with multiple resistance to these botryticides in the field,  
504 although with low frequency, actually could represent a serious threat for typical 'Mazzarrone  
505 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<sub>50</sub> 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<sub>50</sub> values higher than 1 µg ml<sup>-1</sup> (carbendazim, iprodione and pyrimethanil) and 5  
692 µg ml<sup>-1</sup> (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*.