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

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Keywords: Botrytis cinerea; multiple fungicide resistance; table grape; boscalid

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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 SdhB 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.

HIGHLIGHTS

- *Botrytis cinerea* population showed a variable sensitivity level to botryticides
- About 10% of field isolates possessed resistance at least to two or more fungicides
- Different isolates were simultaneously resistant to four different fungicide classes
- No isolates were found resistant to fenexamid and fludioxonil
- Management of resistance in vineyard is needed to delay multiple fungicide breakdown

***Response to Reviewers**

Dear Prof. Stephen N. Wegulo, Principal Editor of Crop Protection Journal,

According to your decision, I have prepared as soon as possible my revised manuscript incorporating the corrections indicated in your attached PDF pages and other minor modification in references and acknowledgments sections.
You can find in the attachment the revised MS by using track-changes method.

Yours sincerely,

Alessandro Vitale

PhD, University of Catania

Dear Prof. S. N. Wegulo, PhD, Crop Protection Editor,

I am writing to you in regard to the manuscript entitled "Detection of field *Botrytis cinerea* isolates with multiple fungicide resistance from table grape in Sicily" that I am submitting to Crop Protection journal for publication.

This manuscript (MS) provides an amount of the data on sensitivity of *B. cinerea,* causal agent of grey mould on grapes, to six fungicides belonging to six chemical groups authorized in Italy for table grape production and worldwide used.
The authors believe that MS could provide useful information to researchers worldwide since several novel findings are reported here:

- The occurrence of field strains with simultaneous resistance to four botryticides belonging to different chemical groups;
- Multiple fungicide resistance, preliminarily detected *in vitro,* has been confirmed by molecular analysis. The breakdown of efficacy was also supported by using a detached grape-berry and grapevine leaf assays; all presented results are referred to an area with a history of severe grey mould attacks on cv. Italia. This production area was recently recognized by European Community with label "Protected and Geographical Indication (PGI) Mazzarrone grape";
- A high degree of sensitivity of *B. cinerea* to fludioxonil and fenhexamid has been detected for all the isolates collected in this area.

The present study provides information about fungicide resistance in commercial vineyards. Since a selection of multi-resistant isolates was detected in our *B. cinerea* population, this study could represent a beginning point for the management of bunch rot on table grape before the control become very difficult. Overall, an effective anti-resistance strategy should be addressed towards the rational application of chemicals. The continuous monitoring of fungicide sensitivity within a cultivation area is a prerequisite for successful disease management, particularly in crops as vineyards, where high grey mold pressure and problems of fungicide resistance have emerged. In view to prolonging the efficacy of fungicides liable to encounter resistance problems and to limit crop losses, this paper provides an evidence of the impact related to use of botryticides mixtures or alternations and stimulate the research in this direction.

We hope that you and Crop Protection editorial board will give full consideration for publication in our journal.

Yours sincerely,

Alessandro Vitale,
PhD, Dipartimento di Agricoltura, Alimentazione e Ambiente - University of Catania

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

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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

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Field Code Changed

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~~ with 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 programmes against grey mould of grape ~~of~~ in 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	Thiophanate-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 EC_{50} value for a boscalid-resistant isolate relative to the EC_{50} 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 above, 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 pathogen-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.

3. Results

3.1. Pathogen sensitivity to fungicides

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

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

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

About 69.2% of the isolates were found sensitive to pyrimethanil (Fig. 1–D), with EC_{50} values 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.

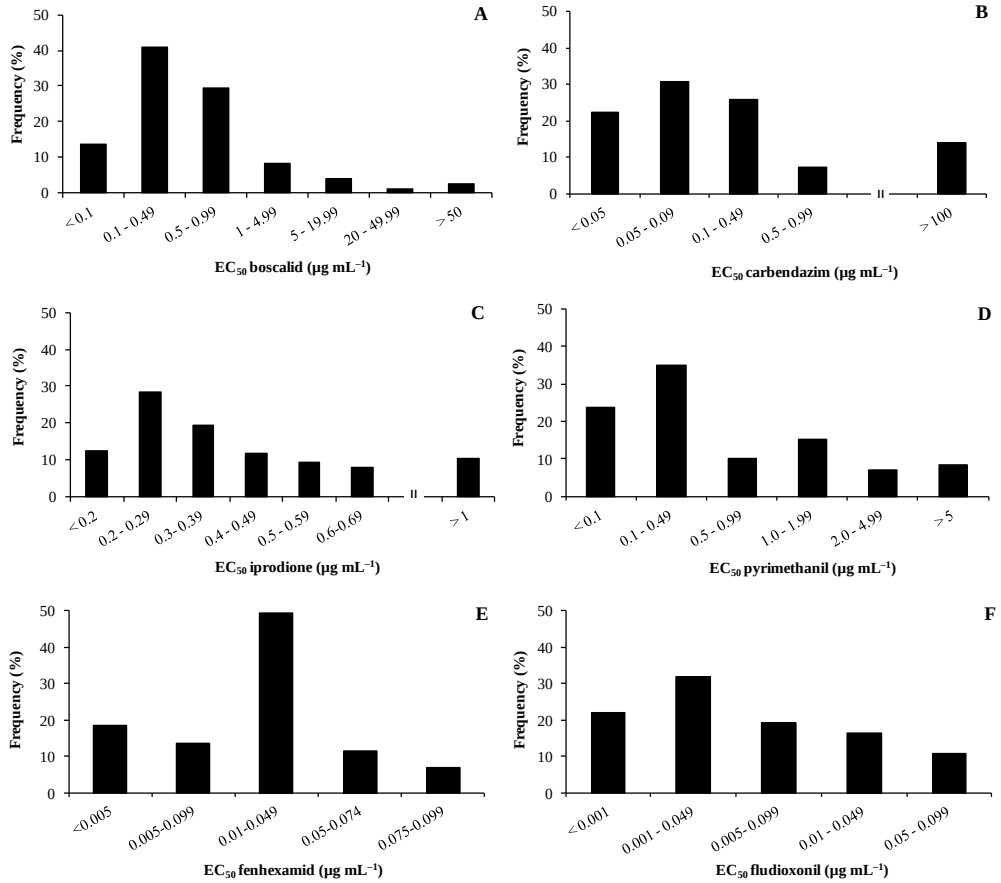

Fig. 1. Frequency distribution of EC_{50} values for boscalid, carbendazim, iprodione, pyrimethanil, fenhexamid and fludioxonil among 302 isolates of *Botrytis cinerea* collected from different vineyards in Sicily.

3.2. Multiple resistance among fungicides

A Venn diagram of sensitivity and resistance to fungicides showed that, among all isolates, 30 isolates exhibited simultaneous *in vitro* resistance to two or more fungicides (Fig. 2). In detail, five isolates were simultaneously resistant to both boscalid and pyrimethanil and twelve to both carbendazim and iprodione. Three isolates were simultaneously resistant to boscalid,

carbendazim and pyrimethanil, two were simultaneously resistant to carbendazim, iprodione and pyrimethanil, whereas eight isolates were simultaneously resistant to boscalid, carbendazim, iprodione and pyrimethanil (Fig. 2, Table 3).

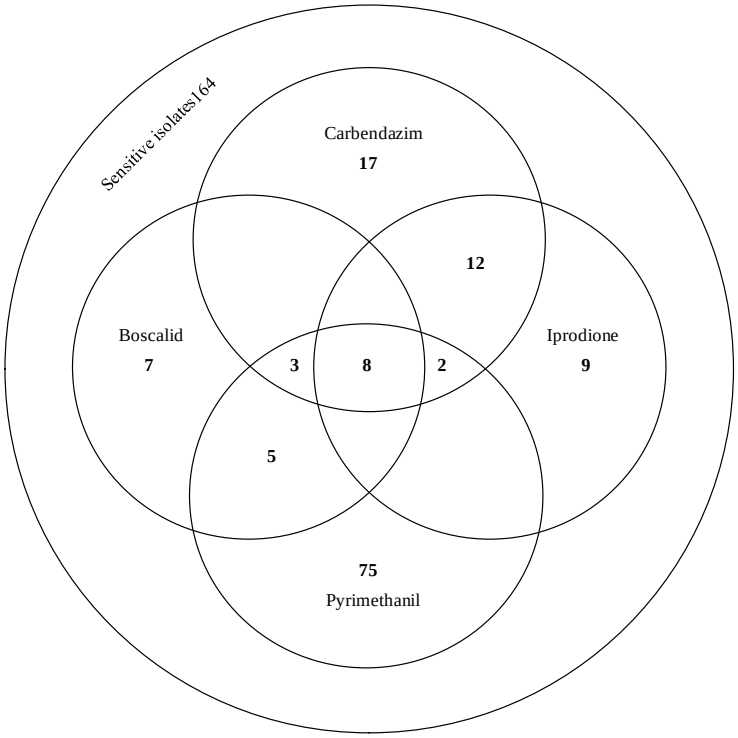

Fig. 2. Venn diagram indicating simultaneous resistance to boscalid, carbendazim, iprodione and pyrimethanil among 302 *Botrytis cinerea* isolates collected from table grape vineyards during 2009-2013. EC_{50} values higher than 1 µg mL^{-1} (carbendazim, iprodione and pyrimethanil) and 5 µg mL^{-1}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

Table 3

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

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
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

[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} values 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 a 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 the BcOS1 gene that confers resistance to the 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)

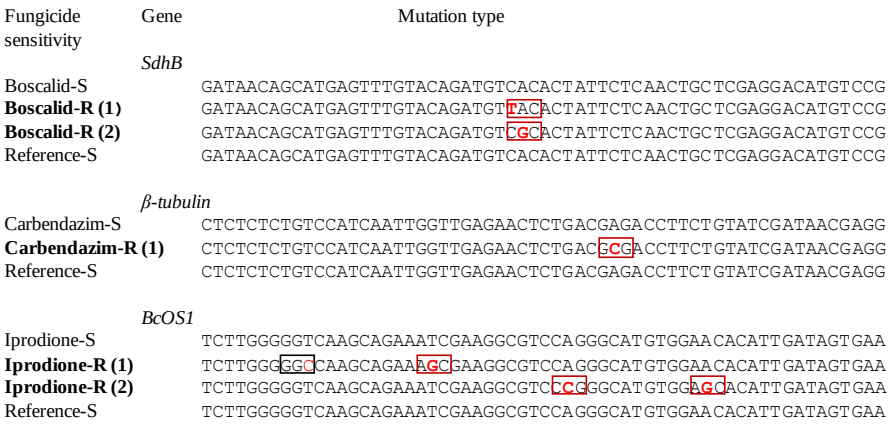

Fig. 3. Different mutations detected in partial nucleotide sequences for SdhB (at codon 272), β-tubulin (at codon 198), and BcOS1 (at codons 365, 369 and 373) genes, respectively, involved in~~to~~ boscalid, carbendazim and iprodione resistance in *Botrytis cinerea*.

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 significantly lower than those detected for R isolates of *B. cinerea*. Moreover, the reductions in lesion size caused by S isolates on fungicide treated grape berries were significant, whereas

373 reductions were not significant for R isolates with the exception of iprodione against isolate
374 MZ4.2 (Table 4).
375 No lesions were observed on pyrimethanil-treated grape berries when S isolates of *B. cinerea*
376 were used for the inoculation. In contrast, pyrimethanil partially failed to control grey mould
377 decay caused by R isolates of *B. cinerea*. Indeed, these latter isolates were able to cause heavy
378 decays on fungicide treated berries (Table 4).
379 Fenhexamid and fludioxonil provided reductions of grey mould decay always higher than 87%
380 and 83%, respectively and no significant differences in the percentage of infected sites were
381 detected among tested isolates (*data not shown*).
382
383 *3.5. Assays on grapevine leaves*
384
385 The ~~R and HR~~isolates that were resistant and highly resistant to boscalid ~~isolates~~ caused
386 visible lesions on grapevine leaves previously treated with the fungicide (Table 4). Indeed, these
387 isolates ~~–~~produced lesions on fungicide-treated leaves which did not significantly differ in
388 diameter from those on control leaves. A low fungicide efficacy in controlling grey mould decay
389 (20.8–41.0% disease reduction) was detected in ~~the RS~~isolates that had reduced sensitivity to
390 boscalid ~~isolates~~. The greatest reductions in disease severity (63.1–100%) were detected in all S
391 isolates.
392 Regarding thiophanate-methyl and pyrimethanil, all isolates considered resistant in previous
393 assays infected fungicide-treated grapevine leaves, producing extensive lesions which were
394 comparable to those observed on untreated controls. No sensitive isolate caused severe symptoms
395 of decay on leaves (disease reduction of 59.6–95.7%).
396 Grapevine leaves treated with iprodione at label rate and then inoculated with sensitive isolates
397 were protected from infection (0.0% of infected sites on treated leaves), whereas those inoculated
398 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~~isolates on grapevine leaves (disease reduction of 92.8–100%) and no significant differences were detected among tested isolates. The diameters of lesions on leaves treated with fungicides and subsequently inoculated with pathogen isolates were significantly ~~lower~~ shorter than those ~~of~~ on 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 Phenotype[a]	Isolates	Infected sites (%)[c]	Lesion (mm) [d] Control	Treated	Reduction (%)	Infected sites (%)[c]	Lesion (mm) [d] Control	Treated	Reduction (%)
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~~ started 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 worldwide (Amiri et al., 2014; Grabke et al., 2013; Leroux, 2007; Saito et al., 2014; Yin et al.,

2014). However, the resistant isolates detected in some studies have only been characterized phenotypically.

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

Currently, field ~~resistant~~ isolates of *B. cinerea* resistant to boscalid have been reported in a limited number of hosts (Amiri et al., 2014; Bardas et al., 2010; Fernández-Ortuño et al., 2014; Veloukas et al., 2011; Yin et al., 2011) including grape in Germany (Wine Road region), France (Champagne region) and, more recently, in Italy (Apulia region) (De Miccolis Angelini et al., 2014; Leroch et al., 2011; Leroux et al., 2010). The low frequency of boscalid-resistant genotypes of *B. cinerea* detected in Sicilian vineyards and conferred by the *Sdh*B$^{H272R/Y}$ mutation, could be due both to its relatively recent introduction (2006 in Italy) and the fact that after the product launch farmers did not use the fungicide frequently, ~~-~~performing a maximum of one application per growing season in recent years. Boscalid–R isolates were detected from all municipalities within the Catania province (Licodia Eubea, Caltagirone and Mazzarrone) although with a very low number per municipality, whereas boscalid-R isolates were collected exclusively in one municipality in Ragusa (i.e. Chiaramonte Gulfi), which incidentally is the most representative for typical grape production in this province. This suggests that the fungicide may yet be included in integrated management programs for control of botrytis bunch rot of 'Mazzarrone grape PGI'. However, the field application of this botryticide should be approached with caution since some pathogen isolates possessed boscalid~~-~~ resistance while other isolates showed an *in vitro* and *in vivo* decreased sensitivity to the fungicide. Therefore, this ~~molecule~~ fungicide should be used in mixtures or in alternations with effective non-cross-resistant fungicides to prolong its lifetime (Zhang et al., 2008).

459 The frequency of benzimidazole-resistant genotypes of *B. cinerea* was found to be relatively
460 low in the detected area and it was associated with the most common worldwide E198V mutation
461 in the β-tubulin gene as reported in other papers (Banno et al., 2008; Ma and Michailides, 2005).
462 This could be partially explained by no or irrelevant use of benzimidazoles in the last decade and,
463 therefore, the almost lack of selection pressure exerted by the fungicide may have induced an
464 increase in wild type (sensitive) isolates having a higher fitness and, consequently, higher
465 competitive activity than resistant isolates. However, the latter isolates could persist within the
466 population for a long time also in the absence of benzimidazole applications (Brent and
467 Hollomon, 2007a).
468 Regarding the dicarboximides, few isolates exhibited resistance to iprodione, showing both the
469 well-known point mutation (type I) at amino acid position 365 (I365S) and amino acid
470 substitutions of type III at position 369 (Q369P) and 373 (N373S) in the histidine kinase genes
471 (*BcOS1*) (Banno et al., 2008). The most dicarboximides-resistant isolates also showed resistance
472 to benzimidazoles, confirming previous data that reported this double resistance in *B. cinerea*
473 populations occurring in a variety of crops (Beever et al., 1989; Brent and Hollomon, 2007a;
474 Yourman and Jeffers, 1999).
475 The high frequency of pyrimethanil-resistant isolates detected in this survey could be related
476 to the widespread use of this fungicide. Resistance to pyrimethanil has developed worldwide and
477 a high percentage of anilinopyrimidine-resistant isolates has been reported in Italy, France,
478 Switzerland, Greece, China and Australia, suggesting that there is a high risk for the occurrence
479 of anilinopyrimidine resistance in *B. cinerea* populations (Baroffio et al., 2003; Chapeland et al.,
480 1999; Gullino et al., 2000; Latorre et al., 2002; Leroux et al., 1999; Myresiotis et al., 2007;
481 Sergeeva et al., 2002; Sun et al., 2010).
482 Regarding fenhexamid and fludioxonil, no fungicide-resistant field isolate was found within
483 our *B. cinerea* population although these compounds have been widely used in Sicilian vineyards.
484 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~~ fungicide 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*~~ *B. cinerea* to fluodioxonil and iprodione confirmed ~~past study~~previous research, 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 area. 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~~ as 'high risk' for resistance

development (EPPO, 2002; Russel, 2004) – due to its polycyclic nature, abundant inoculum production, efficient dissemination mechanisms and wide host range (Myresiotis et al., 2007). Recently, Kretschmer et al. (2009) showed that the mechanism of multiple fungicide resistance for plant pathogens could be additionally due to decreased accumulation of compounds in the mycelium caused by increased fungicide efflux.

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

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References

Abbate, C., Borzì, D., Caboni, P., Baglieri, A., Gennari, M., 2007. Behaviour of fenhexamid in soil and water. J. Environ. Sci. Health - Part B Pestic. Food Contam. Agric. Wastes 42, 843–849.

Amiri, A., Heath, S.M., Peres, N.A., 2013. Phenotypic characterization of multifungicide resistance in *Botrytis cinerea* isolates from strawberry fields in Florida. Plant Dis. 97, 393–401.

Amiri, A., Heath, S.M., Peres, N.A., 2014. Resistance to fluopyram, fluxapyroxad, and penthiopyrad in *Botrytis cinerea* from strawberry. Plant Dis. 98, 532–539.

Banno, S., Fukumori, F., Ichiishi, A., Okada, K., Uekusa, H., Kimura, M., Fujimura, M., 2008. Genotyping of benzimidazole-resistant and dicarboximide-resistant mutations in *Botrytis cinerea* using real-time polymerase chain reaction assays. Phytopathology 98, 397–404.

Bardas, G.A., Veloukas, T., Koutita, O., Karaoglanidis, G.S., 2010. Multiple resistance of *Botrytis cinerea* from kiwifruit to SDHIs, QoIs and fungicides of other chemical groups. Pest Manag. Sci. 66, 967–973.

Baroffio, C.A., Siegfried, W., Hilber, U.W., 2003. Long-term monitoring for resistance of *Botryotinia fuckeliana* to anilinopyrimidine, phenylpyrrole and hydroxyanilide fungicides in Switzerland. Plant Dis. 87, 662–666.

Beever, R.E., Laracy, E.P., Pak, H.A., 1989. Strains of *Botrytis cinerea* resistant to dicarboxamide and benzimidazole fungicides in New Zealand vineyards. Plant Pathol. 38, 427–437.

Brent, K.J., Hollomon, D.W. 2007a. Fungicide resistance in crop pathogens: How can it be managed? FRAC Monograph No. 1 (second, revised edition). Brussels: Fungicide Resistance Action Committee.

561 Brent, K.J., Hollomon, D.W., 2007b. Fungicide resistance: The assessment of risk. FRAC
562 Monograph No. 2 (second, revised edition). Brussels: Fungicide Resistance Action
563 Committee.
564 Billard, A., Fillinger, S., Leroux, P., Lachaise, H., Beffa, R., Debieu, D., 2012. Strong resistance
565 to the fungicide fenhexamid entails a fitness cost in *Botrytis cinerea*, as shown by
566 comparisons of isogenic strains. Pest Manag. Sci. 68, 684–691.
567 Chapeland, F., Fritz, R., Lanen, C., Gredt, M., Leroux, P., 1999. Inheritance and mechanisms of
568 resistance to anilinopyrimidine fungicides in *Botrytis cinerea (Botryotinia fuckeliana).*
569 Pestic. Biochem. Phys. 64, 85–100.
570 De Miccolis Angelini, R.M., Habib, W., Rotolo, C., Pollastro, S., Faretra, F., 2010. Selection,
571 characterization and genetic analysis of laboratory mutants of *Botryotinia fuckeliana*
572 (*Botrytis cinerea*) resistant to the fungicide boscalid. Eur. J. Plant Pathol. 128, 185–199.
573 De Miccolis Angelini, R.M., Rotolo, C., Masiello, M., Gerin, D., Pollastro, S., Faretra, F., 2014.
574 Occurrence of fungicide resistance in populations of *Botryotinia fuckeliana* (*Botrytis*
575 *cinerea*) on table grape and strawberry in south Italy. Pest Manag. Sci. doi: 10.1002/ps-3711.
576 EPPO 2002., EPPO Standard PP 1/213(1) Revision. Resistance Risk Analysis. Bulletin
577 OEPP/EPPO Bulletin.
578 Esterio, M., Auger, J., Ramos, C., García, H., 2007. First report of fenhexamid resistant isolates
579 of *Botrytis cinerea* on grapevine in Chile. (Abstr.). Plant Dis. 91, 768.
580 Faretra, F., Pollastro, S., 1991. Genetic basis of resistance to benzimidazole and dicarboximide
581 fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*). Mycol. Res. 95, 943–951.
582 Fernández-Ortuño, D., Bryson, P.K., Grabke, A., Schnabel, G., 2013. First report of fludioxonil
583 resistance in *Botrytis cinerea* from strawberry field in Virginia. (Abstr.). Plant Dis. 97, 848.
584 Fernández-Ortuño, D., Grabke, A., Bryson, P. K., Amiri, A., Peres, N.A., Schnabel, G., 2014.
585 Fungicide resistance profiles in *Botrytis cinerea* from strawberry fields of seven southern
586 U.S. states. Plant Dis. 98, 825–833.

587 Fillinger, S., Leroux, P., Auclair, C., Barreau, C., Al Hajj, C., Debieu, D., 2008. Genetic analysis
588 of fenhexamid-resistant field isolates of the phytopathogenic fungus *Botrytis cinerea*.
589 Antimicrob. Agents Chemother. 52, 3933–3940.
590 FRAC Code List, 2015: Fungicides sorted by mode of action (including FRAC Code
591 numbering). www.frac.info.com
592 Grabke, A., Fernández-Ortuño, D., Schnabel, G., 2013. Fenhexamid resistance in *Botrytis cinerea*
593 from strawberry fields in the Carolinas is associated with four target gene mutations. Plant
594 Dis. 97, 271–276.
595 Grabke, A., Fernández-Ortuño, D., Amiri, A., Li, X.P., Peres, N.A., Smith, P., Schnabel, G.,
596 2014. Characterization of iprodione resistance in *Botrytis cinerea* from strawberry and
597 blackberry. Phytopathology 104, 396–402.
598 Gullino, M.L., Bertetti, D., Monchiero, M., Garibaldi, A., 2000. Sensitivity to anilinopyrimidines
599 and phenylphyrroles in *Botrytis cinerea* in north-Italian vineyards. Phytopathol. Mediterr. 39,
600 433–446.
601 Hilber, U.W, Schuepp, H., Schwinn, F.J., 1994. Resistance risk evaluation of fludioxonil, a new
602 phenylpyrrole fungicide, in: Heaney, S., Slawson, D., Hollomon D.W., Smith, M,, Russell,
603 P.E., Parry, D.W. (Eds.), Fungicide Resistance. British Crop Protection Council, Farnham,
604 Surrey, pp. 397–402.
605
606 Holz, G., and Volkmann, A. (2002). Colonisation of different positions in grape bunches by
607 potential biocontrol organisms and subsequent occurrence of *Botrytis cinerea*. Bull.
608 IOBC/WPRS 25, 9–12.
609 Hu, M.J., Luo, C.X., Grabke, A., Schnabel, G., 2011. Selection of a suitable medium to determine
610 sensitivity of *Monilinia fructicola* mycelium to SDHI fungicides. J. Phytopathol. 159, 616–
611 620.

612 Kretschmer, M., Leroch, M., Mosbach, A., Walker, A.S., Fillinger, S., Mernke, D., Schoonbeek,
613 H.S., Pradier, J.M., Leroux, P., De Waard, M., Hahn, M., 2009. Fungicide-driven evolution
614 and molecular basis of multidrug resistance in field population of the grey mould fungus
615 *Botrytis cinerea*. PLoS Pathog., 5, e1000696.
616 Lalève, A., Gamet, S., Walker, A.S., Debieu, D., Toquin, V., Fillinger, S., 2013. Site-directed
617 mutagenesis of the P225, N230 and H272 residues of succinate dehydrogenase subunit B
618 from *Botrytis cinerea* highlights different roles in enzyme activity and inhibitor binding.
619 Environ. Microbiol. 16, 2253–2266.
620 Latorre, B.A., Torres, R., 2012. Prevalence of isolates of *Botrytis cinerea* resistant to multiple
621 fungicides in Chilean vineyards. Crop Prot. 40, 49–52.
622 Latorre, B.A., Spadaro, I., Rioja, M.E., 2002. Occurrence of resistant strains of *Botrytis cinerea*
623 to anilinopyrimidine fungicides in table grapes in Chile. Crop Prot. 21, 957–961.
624 Leroch, M., Kretschmer, M., Hahn, M., 2011. Fungicide resistance phenotypes of *Botrytis*
625 *cinerea* isolates from commercial vineyards in south west Germany. J. Phytopathol. 159, 63–
626 65.
627 Leroch, M., Plesken, C., Weber, R.W.S., Kauff, F., Scalliet, G., Hahn, M., 2012. Gray mold
628 populations in German strawberry fields shown multiple fungicide resistance and are
629 dominated by a novel clade close to *Botrytis cinerea*. App. Environ. Microbiol. 79, 159–167.
630 Leroux, P., 2007. Chemical control of *Botrytis* and its resistance to chemical fungicides. In Elad,
631 Y., Williamson, B., Tudzynski, P., Delen, N. (Eds.), Botrytis: Biology,Pathology and
632 Control. Dordrecht: Kluwer Academic, pp. 195–222.
633 Leroux, P., Chapeland, F., Desbrosses, D., Gredt, M., 1999. Patterns of cross-resistance to
634 fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*) isolates from French vineyards. Crop
635 Prot. 18, 687–697.

636 Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., Gredt, M., Chapeland, F.,
637 2002. Mechanisms of resistance to fungicides in field strain of *Botrytis cinerea*. Pest Manag.
638 Sci. 58, 876–888.
639 Leroux, P., Gredt, M., Leroch, M., Walker, A.S., 2010. Exploring mechanisms of resistance to
640 respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold. Appl.
641 Environ. Microbiol. 76, 6615–6630.
642 Ma, Z., Michailides, T.J., 2005. Genetic structure of *Botrytis cinerea* populations from different
643 host plants in California. Plant Dis. 89, 1083–1089.
644 Moyano, C., Gómez, V., Melgarejo, P., 2004. Resistance to pyrimethanil and other fungicides in
645 *Botrytis cinerea* populations collected on vegetable crops in Spain. J. Phytopathol. 152, 484–
646 490.
647 Myresiotis, C.K., Karaoglanidis, G.S., Tzavella-Klonari, K., 2007. Resistance of *Botrytis cinerea*
648 isolates from vegetable crops to anilinopyrimidine, phenylpyrrole, hydroxyanilide,
649 benzimidazole, and dicarboximide fungicides. Plant Dis. 91, 407–413.
650 Myresiotis, C.K., Bardas, G.A., Karaoglanidis, G.S., 2008. Baseline sensitivity of *Botrytis*
651 *cinerea* to pyraclostrobin and boscalid and control of anilinopyrimidine- and benzimidazole-
652 resistant strains by these fungicides. Plant Dis. 92, 1427–1431.
653 Parafati, L., Vitale, A., Restuccia, C., Cirvilleri, G., 2015. Biocontrol ability and action
654 mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch
655 rot of table grape. Food Microbiol. 47, 85–92.
656 Russell, P.E., 2004. Sensitivity baselines in fungicide resistance research and management.
657 FRAC, Monograph No. 3. Brussels: Crop Life International.
658 Saito, S., Cadle-Davidson, L., Wilcox, W.F., 2014. Selection, fitness, and control of grape
659 isolates of *Botrytis cinerea* variably sensitive to fenhexamid. Plant Dis. 98, 233–240.

660 Sergeeva, V., Nair, N.G., Verdanega, J.R., Shen, C., Barchia, I., Spooner-Hart, R., 2002. First
661 report of anilinopyrimidine resistant phenotypes in *Botrytis cinerea* on grapevines in
662 Australia. Australasian Plant Pathol. 31, 299–300.
663 Sun, H.Y., Wang, H.C., Chen, Y., Li, H.X., Chen, C.J., Zhou, M.G., 2010. Multiple resistance of
664 *Botrytis cinerea* from vegetable crops to carbendazim, diethofencarb, procymidone and
665 pyrimethanil in China. Plant Dis. 91, 551–556.
666 Veloukas, T., Leroch, M., Karaoglanidis, G.S., 2011. Detection and molecular characterization of
667 boscalid-resistant *Botrytis cinerea* isolates from strawberry. Plant Dis. 95, 1302–1307.
668 Vitale, A., Cirvilleri, G., Panebianco, A., Epifani, F., Perrone, G., Polizzi, G., 2012. Molecular
669 characterisation and pathogenicity of *Aspergillus* Sect. *Nigri* causing Aspergillus vine canker
670 of table grapes in Italy. Eur. J. Plant Pathol. 132, 483–487.
671 Vitale, A., Panebianco, A., Polizzi, G. Baseline sensitivity and efficacy of fluopyram in *Botrytis*
672 *cinerea* from table grape in Italy. Ann. Appl. Biol. (~~*Submitted*~~*Accepted*).
673 Walker, A.S., Micoud, A., Rémuson, F., Grosman, J., Gredt M., Leroux, P., 2013. French
674 vineyards provide information that opens ways for effective resistance management of
675 *Botrytis cinerea* (grey mould). Pest Manag. Sci. 69, 667–678.
676 Weber, R.W.S., 2011. Resistance of *Botrytis cinerea* to multiple fungicides in Northern German
677 small-fruit production. Plant Dis. 95, 1263–1269.
678 Yin, Y.N., Kim, Y.K., Xiao, C.L., 2011. Molecular characterization of boscalid resistance in field
679 isolates of *Botrytis cinerea* from apple. Phytopathology 101, 986–995.
680 Yin, D., Chen, X., Hamada, M.S., Yu, M., Yin, Y., Ma, Z., 2014. Multiple resistance to QoIs and
681 other classes of fungicides in *Botrytis cinerea* populations from strawberry in Zhejiang
682 Province, China. Eur. J. Plant Pathol. 141, 169–177.
683 Yourman, L.F., Jeffers, S.N., 1999. Resistance to benzimidazole and dicarboximide fungicides in
684 greenhouse isolates of *Botrytis cinerea*. Plant Dis. 83, 569–575.

685 Zhang, C.Q., Liu, Y.H., Zhu, G.N., 2010. Detection and characterization of benzimidazole
686 resistance of *Botrytis cinerea* in greenhouse vegetables. Eur. J. Plant Pathol. 126, 509–515.
687 Zhang, C.Q., Yuan, S.K., Sun, H.Y., Qi, Z.Q., Zhou, M.G., Zhu, G.N., 2007. Sensitivity of
688 *Botrytis cinerea* from vegetable greenhouses to boscalid. Plant Pathol. 56, 646–653.
689 Zhang, C.Q., Zhang, Y., Zhu, G.N., 2008. The mixture of kresoxim-methyl and boscalid, an
690 excellent alternative controlling grey mould caused by *Botrytis cinerea*. Ann. Appl. Biol.
691 153, 205–213.
692 Zhao, H., Kim, Y.K., Huang, L., Xiao, C.L., 2010. Resistance to thiabendazole and baseline
693 sensitivity to fludioxonil and pyrimethanil in *Botrytis cinerea* populations from apple and
694 pear in Washington State. Postharvest Biol. Technol. 56, 12–18.
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696 **Figure Captions**
697
698 **Fig. 1.** Frequency distribution of EC_{50} values for boscalid, carbendazim, iprodione, pyrimethanil,
699 fenhexamid and fludioxonil among 302 isolates of *Botrytis cinerea* collected from different
700 vineyards in Sicily.
701 **Fig. 2.** Venn diagram indicating simultaneous resistance to boscalid, carbendazim, iprodione and
702 pyrimethanil among 302 *Botrytis cinerea* isolates collected from table grape vineyards during
703 2009-2013. EC_{50} values higher than 1 µg ml^{-1} (carbendazim, iprodione and pyrimethanil) and 5
704 µg ml^{-1} (boscalid) classified isolates as resistant and/or with reduced sensitivity to fungicides.
705 The large circle represents the full set of 302 isolates tested for fungicide sensitivity. Each of four
706 smaller circles represents the set of isolates with reduced sensitivity to the corresponding active
707 ingredients. The intersections among different circles indicates 4 subgroups that were
708 simultaneously resistant to more than one fungicide.

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*.