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RESEARCH PAPERS

Characterization of *Botrytis cinerea* populations associated with treated and untreated cv. Moscato vineyards

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Summary. Three *Botrytis cinerea* populations, isolated from three vineyards, one untreated and two treated twice a year, respectively, with fenhexamid or cyprodinil+fludioxonil, were investigated to evaluate the effect of repeated fungicide treatments on the presence and distribution of the transposons *Boty* and *Flipper*, and on the phenotypic traits of each pathogen community. The *vacuma* individuals lacking the two transposons represented the majority of the 390 *B. cinerea* isolates followed by *transposa* strains containing *Boty* and *Flipper*, while the remaining 67 isolates harboured respectively only *Boty* (60) or *Flipper* (7). This research has demonstrated that fungicide application did not influence the transposon distribution patterns, the sensitivity towards various botryticides, or the growth rate of the isolates belonging to the three different populations, but did induced overall reduction of the population size and selected isolates characterized by an enhanced pathogenicity, especially on *Vitis vinifera* leaves.

Key words: transposable elements, grey mould, virulence, phenotype, fungicide.

Introduction

The genus *Botrytis* includes over 20 species, most of which are linked to the sexual stage *Botryotinia,* which in turn is closely related to *Sclerotinia* (Holst-Jensen *et al.,* 1997). The most destructive species in the genus is *Botrytis cinerea* Pers. [synonym *Botryotinia fuckeliana* (De Bary) Whetzel] a worldwide distributed pathogen, which causes grey mould on mature or senescent tissues of more than 200 cultivated and wild host plants (Staats *et al.,* 2005).

On grapevine, bunch rot due to *B. cinerea* induces severe quantitative and qualitative losses: the fungus modifies the chemical composition of berries causing wines to present off-flavors, to be fragile and more sensitive to oxidation and bacterial contamination. Several investigations carried out with molecular markers on populations isolated from different hosts have demonstrated that *B. cinerea* is likely to form a species complex with restricted gene flow among different cryptic groups (Van der Vlugt-Bergmans *et al.,* 1993; Diolez *et al.,* 1995; Kerssies *et al.,* 1997; Giraud *et al.*, 1997, 1999; Alfonso *et al.*, 2000; Yourman *et al.,* 2000; Albertini *et al.,* 2002; Muñoz *et al.,* 2002; Fournier *et al.,* 2003, 2005; Moyano *et al.,* 2003; Ma and Michailides, 2005; Fournier and Giraud 2008; Rajaguru and Shaw, 2010). Populations of *B. cinerea* species complex isolated from different or the same hosts include two sympatric species characterized by the presence, in *transposa* strains, or absence, in *vacuma* strains, of two mobile transposable elements (TE), *Boty* and *Flipper* (Giraud *et al.,* 1999). *Boty* is a 6-kb putative Class I retrotransposon, characterized by a long terminal repeat (LTR), present in multiple copies in different regions of the genome (Diolez *et al.,* 1995; Giraud *et al.,* 1999). *Flipper* is a 1842 bp Class II element, present in up to 20 copies per genome, and is known to be mobile by its insertion into nitrate reductase during spontaneous mutant selection (Levis *et al.,* 1997). Strains containing

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only the *Boty* TE were detected in Europe (Giraud *et al.*, 1999; De Miccolis *et al.*, 2003; Samuel *et al.*, 2012), America (Muñoz *et al.*, 2002; Ma and Michailides, 2005; Esterio *et al.*, 2011) and Australia (Isenegger *et al.*, 2008), while strains harbouring the *Flipper* TE alone have been isolated only in southern and eastern Europe (Albertini *et al.*, 2002; De Miccolis *et al.*, 2003; Milicević *et al.*, 2006; Vaczy *et al.*, 2008; Samuel *et al.*, 2012), Tunisia (Ahmed and Hamada, 2005) and Bangladesh (Isenegger *et al.*, 2008).

Two separate groups, Group I and Group II, were differentiated through analysis of the amino acid polymorphism of two gene products, *Cyp51* (14α-demethylase gene) and Bc-*hch* (*B. cinerea het-c* homolog) (Albertini *et al.*, 2002; Fournier *et al.*, 2003). When first reported, Group I included *vacuma* type isolates resistant to the fungicide fenhexamid and found in France and Germany (Kretschmer and Hahn, 2008), while recently *Boty* TE, coupled with *Flipper* only in a single isolate, was detected in all the Group I strains isolated in Hungary (Fekete *et al.*, 2012). *Botrytis cinerea* populations obtained from different cultivated hosts throughout the world belong almost exclusively to Group II and are generally dominated by *transposa* isolates (Giraud *et al.*, 1997; Topolovec-Pintarić *et al.*, 2004; Ma and Michailides, 2005; Ahmed and Hamada, 2005; Martinez *et al.*, 2005, 2008; Isenegger *et al.*, 2008; Karchani-Balma *et al.*, 2008; Esterio *et al.*, 2011; Fekete *et al.*, 2012). *Vacuma* strains were predominant on green peas in France (Giraud *et al.*, 1999), and apple and kiwifruit in Greece (Samuel *et al.*, 2012), while the great majority of isolates obtained from chickpea in India/Nepal and Bangladesh contained a single TE either *Boty* or *Flipper* (Isenegger *et al.*, 2008). Fertile crosses were obtained between strains within each group, including *vacuma* and *transposa* types in the case of Group II, but not between the groups, indicating that they were reproductively isolated (Fournier *et al.*, 2005). Recently, *B. cinerea* Group I was definitely established as a new species, *B. pseudocinerea* (Walker *et al.*, 2011).

In vineyards, *transposa* strains are predominant (Muñoz *et al.*, 2002; Ma and Michailides, 2005; Kretschmer and Hahn, 2008; Vaczy *et al.*, 2008; Esterio *et al.*, 2011; Samuel *et al.*, 2012), particularly at harvest (Giraud *et al.*, 1997; Martinez *et al.*, 2005), while *vacuma* strains are detected mainly on floral residues and strongly decrease during summer. The higher isolation frequency of *vacuma* strains from senescent tissues has been attributed to marked saprophytic capability linked to rapid growth on highly nutritive media and reduced pathogenicity (Martinez *et al.*, 2005). *Transposa* isolates are characterized by greater virulence on grape berries, and are more frequently resistant to the fungicides vinclozolin and carbendazim (Giraud *et al.*, 1997; Martinez *et al.*, 2003). Phenotypic and molecular diversity has been intensively investigated in *B. cinerea* populations isolated mainly from diseased organs of different plants or at various host developmental stages, but has only seldom been evaluated in strains isolated from untreated or asymptomatic tissues.

Integrated pest management strategies applied in vineyards to reduce grey mould severity include cultural practices (i.e. cultivation of less susceptible varieties, adequate growing and pruning systems, low nitrogen fertilization) and fungicide treatments. Selection pressure exerted by the single site fungicides used against *B. cinerea* population can increase the occurrence of resistant isolates, which, in turn, may lead to a reduced fungicide efficacy.

In the present study, *B. cinerea* populations associated with grape cv. Moscato (very susceptible to the pathogen), in an untreated vineyard and in two plots treated in subsequent vegetative seasons with different active substances, were investigated to assess the effect of repeated fungicide applications on (i) the occurrence and the isolation frequency of the *vacuma* and *transposa* strains, and (ii) the cultural characteristics, pathogenesis and sensitivity toward some commonly used botryticides, of each population.

Materials and methods

Fungal sampling and isolation methods

Samplings were carried out for two consecutive years during the four main vine phenological phases: dormancy, beginning of berry touch, veraison and harvest in three cv. Moscato vineyards located in northern Italy, on the Oltrepo Pavese hills, a few kilometers apart from each other. The vineyards were 20 years old, and trained according to the Guyot system. No herbicides were applied in the vineyards with weed control achieved by mechanical means. Treatments against *Plasmopara viticola* (Berk. *et* Curt.) Berl. and De Toni and *Erysiphe necator* Schwein., the causal agents of downy and powdery mildews, were carried out using fungicides listed in the Integrated

Pest Management (IPM) protocols. For 3 years before the sample collection and during the experimental activity, two vineyards were treated at the beginning of berry touch and 20 d before harvest, one vineyard with fenhexamid (1–1.5 kg ha^{-1} Teldor®, Bayer CropScience), and the other with a mixture of cyprodinil + fludioxonil (0.8 kg ha^{-1} Switch®, Syngenta Crop Protection). The third vineyard was never treated with botryticides. Half a hectare area per vineyard was divided in four blocks, each consisting of three rows of vines. The samples were collected in three subplots constituted of ten vines, located in the central row of each block. In each of the twelve subplots identified in the three vineyards, ten leaves and ten cluster portions, both symptomatic and asymptomatic, were sampled at the different phenological phases, apart from dormancy, when cluster and leaf residues together with lignified shoots were collected. The samples, excised with sterile scissors, were placed in sterile nylon bags, kept at 4°C and rapidly transferred to the laboratory. The asymptomatic organs were incubated in moist chambers at 20°C to induce sporulation. Isolations were performed from sporulating lesions. Conidia were suspended in 1 mL of sterile distilled water, and an aliquot (100 μL) of conidia suspension was spread on a Petri dish on Czapek Yeast Agar (CYA) prepared with 49 g L^{-1} CSA (Czapek Solution Agar, Difco™, Becton Dickinson and Company, Sparks, MD, USA) and 5 g L^{-1} yeast extract (Bacto™ Yeast Extract). After 24 h incubation at 20°C, single germinating conidia were collected and inoculated onto CYA in order to obtain single spore isolates. The isolates were stored both at 4°C on CYA and at -80°C in 20% glycerol/water (v/v).

DNA extraction and genetic characterization

DNA was extracted from *B. cinerea* colonies actively growing on 50 g L^{-1} malt agar (MA) medium (Oxoid Ltd.) overlaid with a sterile cellophane sheet (BCL Cellophane), previously boiled to eliminate plasticizers. After a 48–60 h incubation at 20°C in the dark, 200/300 mg of mycelium from each isolate were frozen with liquid nitrogen and pulverized with a sterile micropestle. DNA was extracted following the protocol described by De Miccolis *et al.* (2010). Solvents and reagents were purchased from Sigma Aldrich Co. The quantity and the quality of the DNA were checked at the spectrophotometer (NanoDrop™ 1000, Thermo Fisher Scientific Inc.).

Isolates belonging to Group I or II were discriminated using a modified version of the method described by Fournier *et al.* (2003). Two primers, Sc1 and Sc2 were designed from the region between position 662 and 1678 of the Bc-*hch* gene (accession number AY032846): Sc1/Sc2: 5′-AACGGCTCACATGGTAAAGG-3′/5′-GCGACTGAAGGAGGTTAGCA-3′. The PCR assay was carried out in a total volume of 20 μL containing 50 ng fungal DNA using Eppendorf *Perfect Taq* DNA polymerase and following the manufacturer's instructions. PCR was performed in an Eppendorf Mastercycler with an initial 3 min denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 60s at 72°C and a 2 min-final extension at 72°C. Amplified products were checked by electrophoresis on 1.5% agarose gel in Tris-acetate (TAE) buffer, stained with ethidium bromide (1 mg L^{-1}) and visualized under UV light (Gel Doc 2000, BioRad Laboratories Inc.). Restriction digestions were performed with restriction Enzime HhaI (Biolabs) following the manufacturer's instructions.

The transposable elements (TEs) *Boty* and *Flipper* were amplified by PCR using specific primer pairs, B/B (Topolovec-Pintarić *et al.*, 2004) and F/F (Giraud *et al.*, 1999) synthesized by PRIMM srl. PCR reactions were carried out according to protocols described by Ma and Michailides (2005). PCR products were separated by electrophoresis on 1.5% agarose gels. Strains carrying *Boty* and *Flipper* TE were classified as *transposa* strains, those with either *Boty* or *Flipper* elements were classified as *Boty* or *Flipper*, while the absence of both TEs characterized the *vacuma* strains.

Phenotypic characterization

The mycelial growth rate, expressed in cm d^{-1}, of all single spore isolates of *B. cinerea* was assessed at three different temperatures: 15, 20 or 25°C on MA, and at 20°C on 39 g L^{-1} potato dextrose agar (PDA) (Difco™). Mycelial plugs (5 mm diam.), excised with a cork borer from the edges of cultures grown on MA for 4 d at 20°C in the dark, were inoculated at the centre of Petri dishes (9 cm diam.) containing MA or PDA. Three replicates per isolate and cultural condition were used. Phenotypic observations concerning the mycelium characteristics, sporulation and sclerotial production of each isolate were carried out daily for 21 d, starting from the day after inoculation, on *B. cinerea* cultures grown on MA at 20°C. As described by Martinez *et al.* (2003), mycelium was

classified in three categories, short (M I), aerial (M II) and showing mycelial masses (M III), while the absence or presence and abundance of sporulation were assessed. The presence, position/distribution and the dimensions of sclerotia were also assessed as: scattered and small (S I), distributed in circles and medium/large (S II), irregular (S III), or undefined distribution or size (S IV).

Virulence assays

Virulence assays were performed on *Vitis vinifera* L. leaves (cv. Cabernet Sauvignon) and berries (cv. Moscato). Inoculations were carried out on the third and the fourth leaf from the shoot apex of cv. Cabernet Sauvignon plants grown in pots in greenhouse at 24°C and 10h:14h light:dark photoperiod. Detached leaves were rinsed twice in sterile distilled water and eight leaf discs (2.5 cm diam.), obtained using a sterile cork borer, were placed in Petri dishes containing 1% water agar (WA) amended with 0.002% kinetin (6-furfurylaminopurine, Sigma Aldrich, Steinheim, CH) to retard leaf senescence. Each leaf disc was inoculated with a mycelial plug (5 mm diam.), excised from the edges of 3-d-old colonies of *B. cinerea* grown on MA and incubated at 20°C in the dark. The inoculated leaf discs were incubated at 20°C for 3 d. The diameter, in cm, of the infected tissues was assessed at the end of the incubation period and results were expressed as percentage of symptomatic area per leaf disc.

Grape bunches of cv. Moscato were collected in the untreated vineyard, at the beginning of berry touch, veraison or harvest. Detached berries, rinsed twice in sterile distilled water, were placed in the 24 wells of multi-well plates (Falcon, Becton Dickinson and Company) containing WA and kinetin, and were each wounded with a sterile inoculation needle. Each *B. cinerea* strain was tested by inoculating the injured tissues of eight damaged berries with a mycelial plug (5 mm diam.), obtained as previously described. The plates were incubated at 20°C for 3 d. After incubation, disease symptoms were evaluated according to the following visual semi-quantitative scale: 0 = healthy; 1 = symptoms visible on 10% of the berry surface; 2 = symptoms visible on 25% of the berry surface; 3 = symptoms visible on 50% of the berry surface; 4 = symptoms visible on 75% of the berry surface; 5 = symptoms visible on 100% of the berry surface; 6 = symptoms visible on 100% of the berry surface associated with sporulation. A percentage infection index (I%I) was calculated according to the formula proposed by Townsend and Heuberger (1943).

Fungicide sensitivity assay

The fungicide sensitivity assays were performed by estimating the fungal biomass with an automated quantitative method, using a conidial suspension of each *B. cinerea* isolate at 1×10^2 conidia mL^{-1} concentration (Raposo *et al.*, 1995). Each isolate was cultured on CYA for 7 days at 20°C in the dark, and a conidial suspension was prepared in sterile 0.01% Tween 20 (Merck KgaA). Conidial concentration was adjusted to obtain the final concentration of 1×10^4 conidia mL^{-1} in 20% glycerol:water solution and then stored at -20°C. The fungicides, and the corresponding concentrations tested against the isolates, are listed in Table 1. The highest concentration of each fungicide corresponds to the ED_{50}, as determined in previous investigations (Leroux *et al.*, 1999; Moyano *et al.*, 2004; Takagaki *et al.*, 2004; Stammler and Speakman, 2006; Myresiotis *et al.*, 2007). Depending on the results of the sensitivity assay, decreasing concentrations of all fungicides, apart from boscalid, vinclozolin and carbendazim, were tested in order to obtain an average 50% growth inhibition of the *B. cinerea* population associated with cv. Moscato in northern Italy. The fungicides were dissolved in dimethylsulphoxide (DMSO, Carlo Erba Reagenti) at a maximum 0.3% (v:v) final concentration in the growth medium utilized for the sensitivity assays, since preliminary assays carried out with numerous isolates demonstrated that this DMSO concentration did not inhibit fungal growth.

The sensitivity assays were carried out in 96 well microtiter plates (Sero-wel, Bibby Sterilin Ltd), using sterilized minimal cultural medium (MCM, Myresiotis *et al.*, 2007). Yeast extract was omitted in anilinopyrimidine assays, to rule out interferences with their mode of action (Masner *et al.*, 1994). The assays were performed in triplicate. Absorbance of each well was measured at 492 nm in a Sunrise Absorbance Reader (Tecan Group Ltd) immediately before and after the incubation period (3 d at 20°C in the dark). Growth inhibition percentage (GIP) was calculated according to the following formula: $[(AC_{t3}-AC_{t0})-(AF_{t3}-AF_{t0})]\times100/(AC_{t3}-AC_{t0})$; where AC_{t0} and AF_{t0} are respectively, the initial absorbances of control (C) and fungicide (F) treated samples, and AC_{t3} and AF_{t3} are the final absorbances.

Table 1. Concentrations and classes of the active substances tested in fungicide sensitivity assays. The concentration of each fungicide inducing an average 50% growth inhibition reported in literature is included in bold font.

Active substance	Concentration (mg L^{-1})	Fungicide class
Pyrimethanil	**0.7**, 0.3, 0.1, 0.05, 0.01	Anilinopyrimidine
Cyprodinil	**0.03**, 0.01, 0.007	Anilinopyrimidine
Mepanipyrim	**3.0**, 1.0, 0.6, 0.4	Anilinopyrimidine
Fludioxonil	**0.07**, 0.01	Pyrrole
Fenhexamid	**0.1**, 0.01	Hydroxyanilide
Boscalid	**0.06**	Pyridine- carboxamides
Vinclozolin	**0.5**	Dicarboxamide
Carbendazim	**0.01**	Benzimidazole

Statistical analyses

The isolation frequencies of mycelial and sclerotial strains and of *vacuma*, *transposa*, *Boty* and *Flipper* strains isolated from different sampling sites and at different phenological phases were analyzed using χ^2 test with Yates' correction for continuity. GIPs of the four molecular types in the three vineyards were compared by a non-parametric Kruskal-Wallis test. Growth rate, virulence and fungicide sensitivity data were compared by one way ANOVA and multiple comparisons of the mean values by Tukey's test. Where necessary, percentage data were converted into Bliss angular values (arcsin√%) to meet ANOVA assumptions (normal distribution as assessed by Shapiro-Wilk test [$P>0.05$] and homogeneity of variance by Levene's test [$P>0.05$]). The Pearson coefficient (r) was used to estimate correlation between virulence on leaves and berries, between virulence on different host organs, and mycelial growth rate and cross resistance patterns between fungicides. All the statistical analyses were performed using SPSS Statistics software (version 18, IBM).

Results

Botrytis cinerea strains

Overall, 390 *B. cinerea* single spore isolates were obtained from cv. Moscato during two consecutive years from the untreated and two treated vineyards (Table 2). Based on the number of isolates, the organs collected in the untreated vineyard were more contaminated by the pathogen than the corresponding plant material sampled in the treated vineyards (Table 2), with the exceptions of i) the leaves of both treated vineyards at the beginning of berry touch, ii) the rachises collected in the fenhexamid treated vineyard at veraison and iii) ripe asymptomatic berries collected at harvest in the vineyard treated with cyprodinil + fludioxonil.

Botrytis cinerea isolates were obtained mainly at harvest (44%) and, with decreasing frequency, at dormancy (23%), beginning of berry touch (17%) and veraison (16%).

The majority of isolates (59%) was obtained from berries, either asymptomatic (156 isolates) or symptomatic (76 isolates). The remaining isolates were derived from leaf and cluster residues (18%), leaves (16%), less frequently from shoots (5%) and rachises (2%).

Molecular characterization

The digestion (with the restriction enzyme *Hha*I) of the 1017 bp fragment of the Bc-*hch* gene, corresponding to the region homologous to the *het-c* gene that regulates vegetative compatibility in *Neurospora crassa* (Fournier *et al.*, 2003), showed a single restriction pattern corresponding to Group II (*B. cinerea sensu stricto*).

The frequency of *vacuma* and *transposa* isolates, which were the more abundant molecular types, var-

Table 2. Numbers of *Botrytis cinerea* isolates obtained from different grapevine berries, leaves, rachises, shoots and residues in the main phenological phases from different vineyards.

Phenological phase	Organ	Vineyard			Total
		Untreated	Fenhexamid	Cyprodinil + fludioxonil	
Dormancy	Residues	26	20	23	88
	Shoot	12	3	3	
	Rachis	1	0	0	
Beginning of berry touch	Berry	23	11	12	68
	Leaf	6	9	7	
Veraison	Berry	17	7	17	63
	Leaf	14	1	2	
	Rachis	1	4	0	
Harvest	Berry	47	39	61	171
	Leaf	18	3	3	
Total		165	97	128	390

ied with the vineyard, whereas analogous percentages of *Boty* (11–18%) and *Flipper* (2%) isolates were found in the three populations (Figure 1). *Vacuma* strains prevailed on *transposa* in the untreated and in cyprodinil and fludioxonil treated vineyards, whereas in the vineyard treated with fenhexamid the frequency of the two molecular types was similar. The proportion of both *vacuma* and *transposa* found in the untreated plot and cyprodinil+fludioxonil treated vineyards was similar ($\chi^2 = 0.27$; df = 1; $P = 0.6$), while significant differences were found between the untreated and the fenhexamid treated plots ($\chi^2 = 4.21$; df = 1; $P = 0.04$). *Vacuma* strains were isolated more frequently in comparison with the other molecular types at all host phenological phases, ranging from 49% during the beginning of berry touch to 60% at veraison (Table 3). The isolation frequency of *transposa* strains ranged from 21% at the beginning of berry touch to 37% at harvest. Isolates carrying the *Boty* TE were obtained at all phenological phases with frequencies varying from 10% (harvest) to 30% (beginning of berry touch). On the contrary, *Flipper* isolates were obtained at veraison only in the untreated vineyard, and at harvest, in all three vineyards. Significant differences were detected in the distribution of the molecular types at the different phenological phases ($\chi^2 = 26.69$; df = 9; $P = 0.01$) but not in their isolation frequency from berries, leaves, rachises and shoots.

Morphological characterization and growth rate

No differences were detected in the distribution of the four molecular types in the two morphological classes, mycelial or sclerotial. The great majority of the mycelial type isolates (43% of the total isolates) was able to form aerial mycelium and to sporulate, while the sclerotial isolates (57%) did not form any conidia on the culture media. Significant differences in the isolation frequencies of mycelial and sclerotial isolates were detected between the untreated and the cyprodinil and fludioxonil treated vineyards ($\chi^2 = 9.06$; df = 1; $P = 0.003$), while analogous results were obtained in the untreated and the fenhexamid treated vineyards ($\chi^2 = 0.001$; df = 1; $P = 0.98$).

While similar percentages of mycelial and sclerotial strains were found in the untreated (46% mycelial type, 54% sclerotial type) and in the fenhexamid treated vineyards (45% mycelial type, 55% sclerotial type) (Figure 2), in the vineyard treated with cyprodinil and fludioxonil, a greater number of sclerotial isolates were obtained (36% mycelial type, 64%

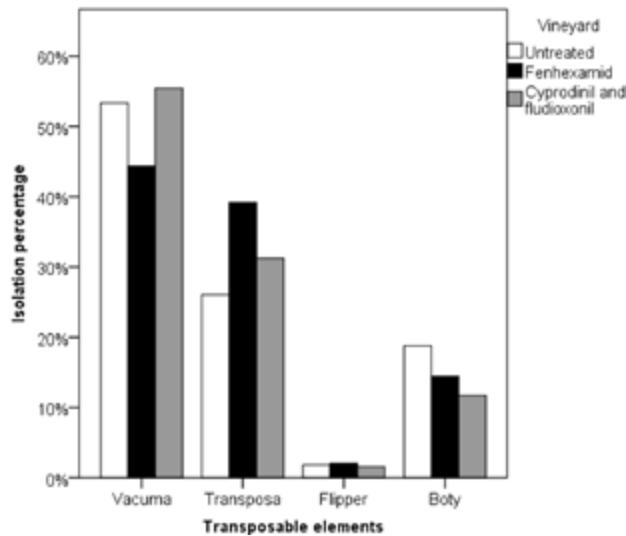

Figure 1. Isolation frequency of the *Botrytis cinerea* strains belonging to the four molecular types from different sampling sites.

sclerotial type), and the percentage of sporulating strains was lower (38%) than in the other sampling sites (55%).

The isolation frequencies of the mycelial or sclerotial isolates varied with the grapevine phenological stages, but did not show significant differences. Sclerotial isolates were predominant in samples collected during dormancy and at harvest.

The growth rate of the 390 isolates was influenced by the incubation temperature, the culture medium and the molecular type. On MA, the daily average growth rates assessed at the three different incubation temperatures were significantly different (F = 222.2; df = 2; P<0.0001): the greatest and least values were observed, respectively, at 20°C (1.67 cm d^{-1}), and at 15°C (1.26 cm d^{-1}), while at 25°C the average daily growth rate was 1.56 cm d^{-1}. At 20°C, the average daily growth rate of the isolates was significantly higher (F = 1119; df =1; P<0.0001) on PDA (2.5 cm d^{-1}) than on MA.

Apart from on MA at 15°C, the isolates from the untreated vineyard generally showed significantly higher average daily growth rates in comparison with isolates collected in the treated plots (Figure 3). The latter individuals were characterized by similar average daily growth rates on MA and PDA at all the tested temperatures. While mycelial and sclerotial isolates showed analogous average daily growth rates on MA at all the tested temperatures, significant differences were found on PDA at 20°C: mycelial isolates (2.6 cm d^{-1}) had significantly higher average daily growth rates (F = 7.93; df = 1; P=0.005) than sclerotial isolates (2.5 cm d^{-1}). The mycelial growth rate of the isolates characterized by the presence of one or two transposable elements and *vacuma* isolates on MA was not significantly different. On PDA, the daily growth rates of *vacuma* strains were significantly faster (2.6 cm d^{-1}) than the other molecular types (F = 4.4; df = 3; P=0.004). *Transposa* and *Boty* strains exhibited the same average daily growth rate (2.4 cm d^{-1}), whereas the strains carrying the *Flipper* TE alone showed the lowest average daily growth rate (2.2 cm d^{-1}).

Virulence

Virulence on leaves

Botrytis cinerea showed variable virulence with percentage of symptomatic area ranging from 0 to 91% on detached leaves of *V. vinifera* cv. Cabernet Sauvignon (Figure 4a).

Table 3. Numbers (and frequencies) of *Botrytis cinerea transposa* and *vacuma* strains and isolates characterized by *Boty* or *Flipper* elements obtained from different grapevine phenological phases.

Phenological phase	*Transposa*	*Boty*	*Flipper*	*Vacuma*	Total
Dormancy	28 (32%)	14 (16%)	0	46 (52%)	88
Beginning of berry touch	14 (21%)	21 (30%)	0	33 (49%)	68
Veraison	15 (24%)	8 (13%)	2 (3%)	38 (60%)	63
Harvest	64 (37%)	17 (10%)	5 (3%)	85 (50%)	171
Total	121	60	7	202	390

The mean proportion of leaf area infected was 16% for the isolates from the untreated vineyard, 27% for isolates from the fenhexamid treated vineyard and 28% for isolates from the cyprodinil + fludioxonil treated vineyard. The isolates obtained from the untreated vineyard showed significantly lower ability to cause disease symptoms on leaves (F = 28.81; df = 1; P<0.0001). No differences in virulence were detected between the isolates obtained from the two fungicide treated vineyards.

Significant differences in the virulence of isolates collected at various host phenological phases were detected: isolates obtained during veraison were significantly less virulent on leaves (15% of leaf infected area) in comparison with those collected during other phenological phases (20% at berry touch, 25% at harvest). Isolates obtained during dormancy showed the highest average percentage of symptomatic leaf area (30%, F = 7.23; df = 3; P<0.0001).

Virulence on leaves was influenced neither by the mycelial nor sclerotial growth, nor by the presence of TE.

Virulence on berries

The virulence of the *B. cinerea* isolates also varied greatly on berries (Figure 4b). All the strains caused grey mould in berries collected at harvest (I%I range = 2–69%), but not at the beginning of berry touch (I%I range = 0–71%) or at veraison (I%I range = 0–75%). Two isolates obtained in winter from the fenhexamid treated vineyard did not induce any necrotic lesions on leaves or berries collected at the beginning of berry touch, while nine isolates from treated and untreated vineyards obtained during harvest and dormancy did not cause any disease symptoms on berries collected at veraison.

The susceptibility of berries was significantly influenced by their growth stage (F = 50.98; df = 2; P<0.0001): the highest average I%I (48.7%) was observed on berries collected at veraison, an intermediate value was assessed on berries sampled at harvest (39.3%), whereas berries collected at the beginning of berry touch showed even lower susceptibility to the pathogen (36.6%). The I%I assessed on berries collected at veraison is positively correlated with those assessed on the same organs collected at the beginning of berry touch (r = 0.17, P=0.006) and at harvest (r = 0.14, P=0.004).

No differences in virulence on berries collected in the three phenological phases were detected between isolates obtained from different sampling sites, between mycelial and sclerotial strains and the four molecular types. On the contrary, isolates ob-

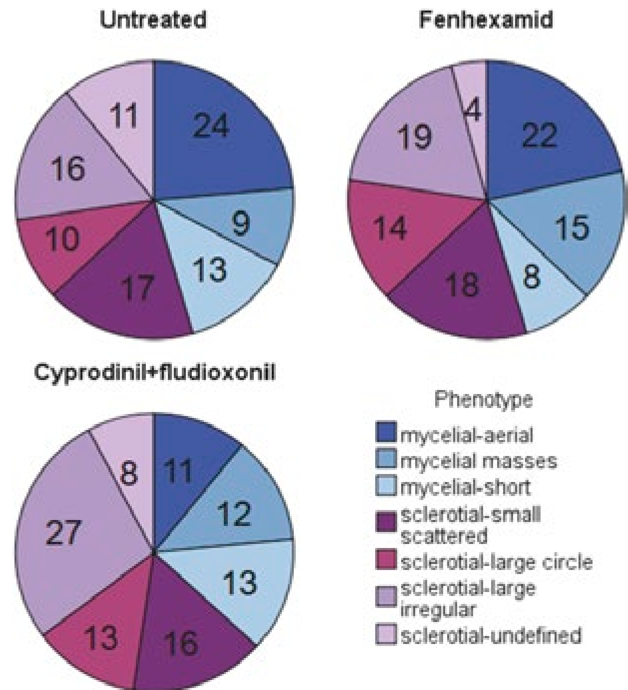

Figure 2. Phenotypic classification of *Botrytis cinerea* strains isolated from untreated and treated vineyards.

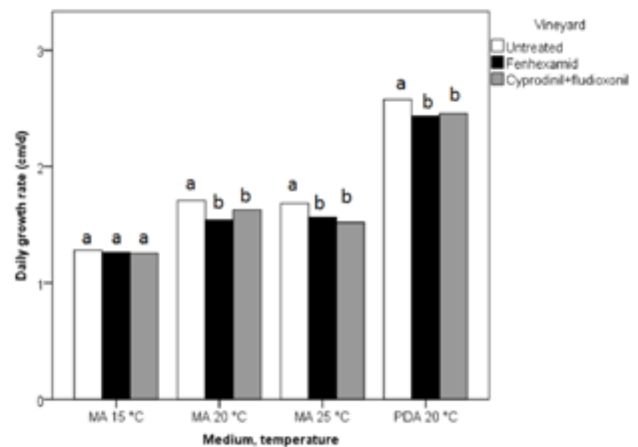

Figure 3. Daily average growth rates (cm d^{-1}) of *Botrytis cinerea* isolates obtained from untreated or fungicide treated vineyards on two agar media and at three different temperatures (15, 20 or 25°C). Within each of the experimental conditions, bars with different letters are significantly different according to the Tukey's test after ANOVA (P<0.05).

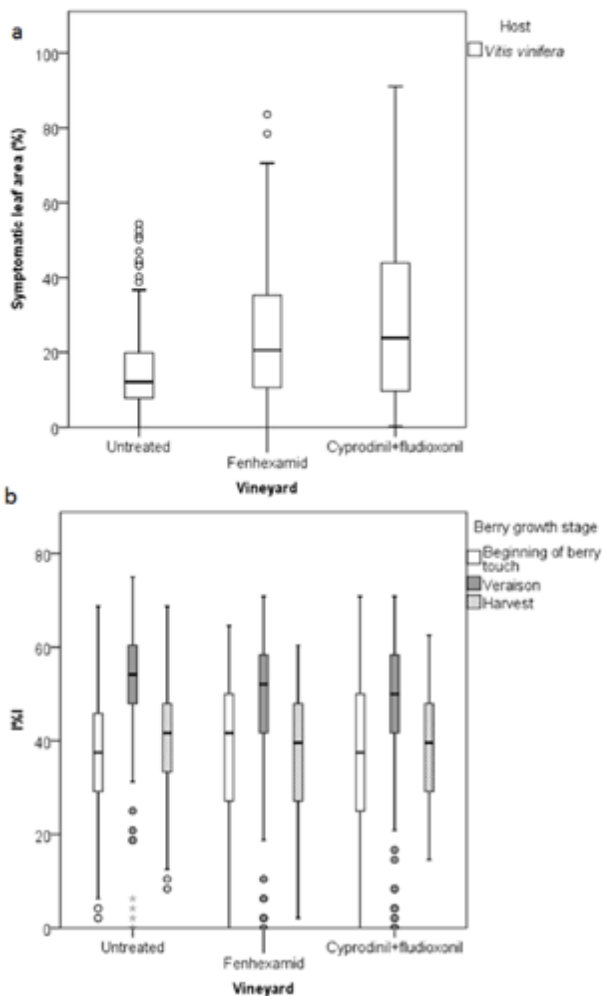

Figure 4. Box plots[a] of virulence distribution of *B. cinerea* strains isolated from untreated and treated vineyards. (a) percentage of symptomatic leaf area on *V. vinifera* leaves (b) percentage infection index (I%I) on *V. vinifera* berries.

[a]The box plots depict the five-number summaries, namely the minimum and maximum values, the upper and lower quartiles and the median. The median is identified by a line inside the box. The length of the box represents the interquartile range (IQR). Values more than 3 IQRs from either end of the box are labeled as extremes and are denoted by an asterisk (*). Values more than 1.5 IQRs but less than three IQRs from either end of the box are labeled as outliers (O).

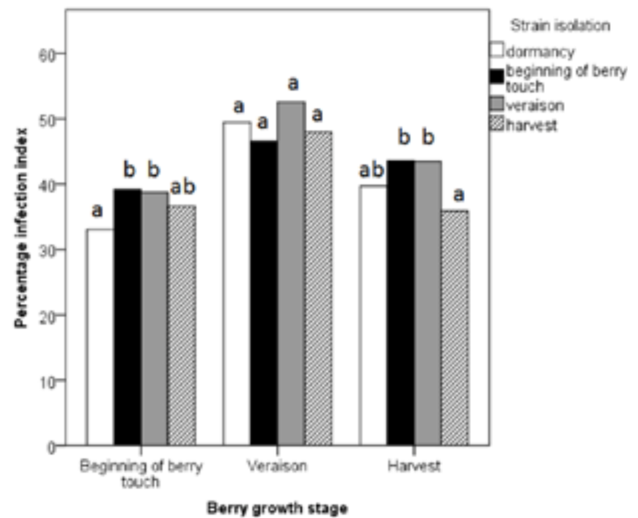

Figure 5. Virulence of *Botrytis cinerea* isolates obtained during the main phenological phases, on berries collected at the beginning of berry touch, veraison and harvest. Means with different letters are significantly different according to the Tukey test after ANOVA ($P<0.001$)

tained from samples collected during dormancy and at harvest induced significantly lower I%I values ($F = 10.23$; df = 3; $P<0.001$) on berries collected at beginning of berry touch and at harvest in comparison with isolates obtained from different grapevine organs in the two remaining phenological phases. No differences in virulence were observed on berries collected during veraison (Figure 5).

Isolates obtained from the reproductive and vegetative grapevine organs did not show significant differences in virulence on leaf discs and berries.

The average daily growth rate of the isolates assessed on PDA at 20°C is negatively correlated with their corresponding virulence on leaves ($r = -0.2$, $P<0.001$), and positively with the I%I detected on berries collected at veraison ($r = 0.2$, $P=0.004$).

Fungicide sensitivity assays

The doses of each tested fungicide and the corresponding average inhibition percentage of the *B. cinerea* population associated with cv. Moscato are shown in Table 4. The 390 isolates showed highly variable growth inhibition percentages (GIPs) at the tested fungicide doses (Figure 6). No differences in the growth inhibition distributions were detected for isolates obtained from untreated or treated vineyards, apart for the fungicides vinclozolin and mepanypirim.

The GIPs of the 390 isolates due to the carboxamide fungicide boscalid added at 0.06 mg L^{-1} to the

Table 4. Fungicide doses and average growth inhibition percentages (GIP) of the *Botrytis cinerea* isolates obtained from grapevine cv. Moscato in northern Italy.

Fungicide	Concentration (mg L^{-1})	GIP (%)
Boscalid	0.06	54.3
Vinclozolin	0.5	55.9
Carbendazim	0.05	63.8
Fenhexamid	0.01	46.2
Cyprodinil	0.007	61.3
Mepanipyrim	0.4	55.7
Pyrimethanil	0.05	64.4
Fludioxonil	0.01	41.2

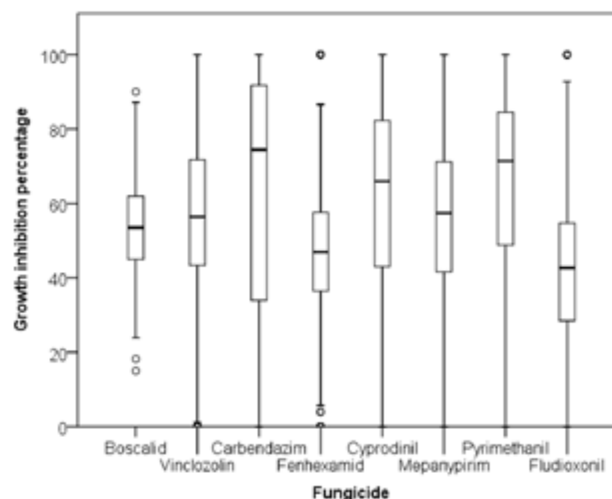

Figure 6. Box-plot distribution of the growth inhibition percentage (GIPs) of *Botrytis cinerea* isolates incubated with boscalid, vinclozolin, carbendazim, fenhexamid, cyprodinil, mepanypyrim, pyrimethanil or fludioxonil. Values more than 1.5 IQRs but less than three IQRs from either end of the box are labeled as outliers (○).

cultural medium, ranged from 15 to 90% (Figure 6). No totally insensitive or totally inhibited isolates were detected, and the central part of the distribution ranged between 45 and 62% with a 53% median. The three outliers were represented by a single isolate showing the highest GIP due to boscalid (90%), obtained from the untreated vineyard, and two individuals only slightly inhibited by boscalid, obtained from the fenhexamid treated vineyard at the beginning of berry touch.

A generally wide distribution of the GIPs, ranging from 0 to 100%, with 56% median and mean, characterized the dicarboximide fungicide vinclozolin (Figure 6). The strains isolated from the untreated vineyard (GIP = 66.5%) were significantly more sensitive than those collected from the fungicide treated vineyards (GIP fenhexamid = 46.4%; GIP cyprodinil and fludioxonil = 49.3%) ($F = 34.41$; df = 2; $P<0.0001$). Moreover, all the 13 isolates that were completely unaffected by vinclozolin were isolated from the treated vineyards.

The benzimidazole fungicide carbendazim, added to the culture medium at 0.005 mg L^{-1}, induced GIP values varying from 0% to 100%, but 75% of the individuals showed GIPs higher than 35% (Figure 6). The majority of the 30 isolates totally inhibited by carbendazim was isolated from the untreated vineyard, while 15 of the 17 insensitive isolates were obtained from the treated plots.

Fenhexamid caused GIP values ranging from 5% to 88%, but some outliers were identified (Figure 6). In particular, eight isolates, one collected in the vineyard treated with the anilide fungicide and the others from the untreated vineyard, were completely inhibited by fenhexamid at 0.001 mg L^{-1}. On the contrary 19 isolates, three obtained from the untreated vineyard, ten from the fenhexamid and six from the cyprodinil and fludioxonil treated vineyards, were completely unaffected by the tested doses of the fungicide.

The GIP value for the anilinopyrimidine fungicides cyprodinil, mepanypirim and pyrimethanil ranged from 0% to 100%, with 75% of the individuals strongly affected by the fungicide (GIP > 40%) (Figure 6). Cyprodinil, in particular, completely inhibited the growth of 21 isolates: 11 collected from the treated vineyards, and ten from the untreated vineyard. On the contrary, 25 isolates, 18 obtained from both the fungicide treated vineyards at dormancy, veraison and harvest, and seven collected from the untreated vineyard, were unaffected by cyprodinil, and were also insensitive to pyrimethanil at 0.05 mg L^{-1}. The same dose of pyrimethanil caused complete growth inhibition of 37 isolates: 26 collected from the two fungicide treated vineyards and 11 from the untreated vineyard. Among the 28 insensitive strains, 25 were unaffected also by cyprodinil. Only three

isolates obtained from samples collected in the two treated vineyards were completely inhibited by 0.4 mg L^{-1} mepanypirim. The eight isolates insensitive to the assessed doses of mepanypirim were obtained from plant organs collected in both the differently treated vineyards and none of these strains were simultaneously insensitive to the other tested active ingredients. Isolates collected from the treated vineyards (GIP fenhexamid = 61%; GIP cyprodinil and fludioxonil = 59%) were significantly more inhibited by mepanypirim (*F* value = 9.28; df = 2; *P*=0.0001) than isolates obtained from the untreated vineyard (GIP = 50%).

A wide distribution of GIPs, characterized by values ranging from 0 to 93% and a median of 43%, also characterized the pyrrole fungicide fludioxonil (Figure 6). At the tested doses, fludioxonil caused the total growth inhibition of six isolates, obtained in the same number from the vineyard treated with fenhexamid and from the untreated plot, and was completely ineffective on 30 strains: 12 collected from the untreated vineyard and 18 from the two fungicide treated vineyards.

Only six isolates were simultaneously insensitive to different fungicides ingredients: two isolates obtained respectively from the untreated and the cyprodinil + fludioxonil treated plots, were unaffected by both vinclozolin and carbendazim, and two isolates, obtained respectively from the fenhexamid treated vineyard and from the untreated plot, were unaffected by fenhexamid and carbendazim. Of these last two strains collected in the anilide treated plot and insensitive to fenhexamid, one was resistant to vinclozolin and the other to pyrimethanil and carbendazim.

Correlation coefficients between all the inhibition values due to the tested fungicides did not show significant results, apart from a few cases concerning the two anilinopyrimidine fungicides pyrimethanil and cyprodinil (r = 0.5; P<0.001), boscalid and vinclozolin (r = 0.4; P<0.001) and vinclozolin and fenhexamid (r = 0.3; P=0.001).

No significant differences in the sensitivity to fungicides used at the doses indicated in Table 4 were found between strains collected at the different host phenological phases and between mycelial and sclerotial strains. Virulence on leaves and berries and sensitivity to the various fungicides were not related.

According to the Kruskal-Wallis test, the four molecular types isolated from individual vineyards did not differ in the distribution of GIPs induced by all the tested fungicides (Table 5), apart from a single case: *Flipper* strains isolated from the fenhexamid treated vineyard showed a significant GIP reduction (*P*=0.044) caused by cyprodinil (GIP = 37%). In addition, even though not significantly different from the other molecular types, *Flipper* strains isolated from the cyprodinil + fludioxonil treated vineyard showed a reduced range in GIP distribution caused by cyprodinil (29–30%) (Figure 7a) and boscalid (60–61%) (Figure 7b) in comparison with the other molecular types.

Discussion

All *B. cinerea* strains isolated from untreated and treated vineyards of cv. Moscato in northern Italy belonged to Group II, as already observed in other vineyards located in Tunisia (Ahmed and Hamada, 2005; Karchani–Balma *et al.*, 2008), Greece (Samuel *et al.*, 2012) and Chile (Esterio *et al.*, 2011). Group I strains, now belonging to *B. pseudocinerea*, up to now were sporadically detected only in Europe, in France and Germany on grapevine leaves and blossoms and in Hungary on raspberry and rape (Martinez *et al.*, 2005; Fekete *et al.*, 2012). Since no Group I strains were isolated from cv. Moscato, it seems that *B. pseudocinerea* is epidemiologically irrelevant in northern Italian vineyards.

Fungicide treatments carried out on cv. Moscato against *B. cinerea* reduced the pathogen occurrence on shoots and particularly on leaves, while no difference in berry contamination was found between the untreated and the cyprodinil + fludioxonil treated plots. However the cyprodinil + fludioxonil treatment resulted mainly in latent infections, probably due to the anilinopyrimidine fungicide ability to affect mycelial development of the pathogen through the inhibition of secretion of hydrolytic enzymes. The samples collected in the untreated vineyard showed the greatest contamination levels, in particular during dormancy and at the beginning of berry touch. In the three examined plots, the berries, particularly the ripen ones, were the principal sources of *B. cinerea* isolates.

Treatment application on cv. Moscato affected the isolation frequency of the two main molecular types *transposa* and *vacuma* only in the fenhexamid treated vineyard, but did not influence the presence of *Boty* and *Flipper* strains. However, the frequency pattern

Table 5. Average fungicide growth inhibition percentages (GIPs) for four *Botrytis cinerea* molecular types isolated from the differentially treated vineyards.

Vineyard	Fungicide	Transposable elements			
		Vacuma	***Transposa***	***Flipper***	***Boty***
Untreated	Boscalid	55.2	55.9	50.8	57.9
	Vinclozolin	64.8	69.7	59.1	67.7
	Carbendazim	68.9	54.5	52.1	56.6
	Fenhexamid	49.8	48.2	46.9	48.0
	Cyprodinil	61.4	66.4	53.6	63.8
	Mepanypirim	47.1	53.9	35.8	55.0
	Pyrimethanil	62.9	62.7	59.0	71.3
	Fludioxonil	41.8	42.8	39.5	40.3
Fenhexamid	Boscalid	53.7	51.9	51.1	53.3
	Vinclozolin	51.4	38.6	52.9	51.3
	Carbendazim	56.5	62.8	61.3	67.2
	Fenhexamid	43.8	38.2	24.5	44.9
	Cyprodinil	50.7	66.0	37.3	69.1
	Mepanypirim	59.1	65.4	47.0	55.9
	Pyrimethanil	61.5	72.4	89.8	62.3
	Fludioxonil	44.5	43.1	34.7	29.4
Cyprodinil + fludioxonil	Boscalid	52.7	51.8	60.8	58.1
	Vinclozolin	50.2	46.4	54.3	52.1
	Carbendazim	71.1	61.5	74.9	67.4
	Fenhexamid	47.4	43.2	40.2	48.9
	Cyprodinil	59.9	60.0	29.6	48.4
	Mepanypirim	57.1	57.7	62.8	71.0
	Pyrimethanil	60.7	68.5	42.8	60.9
	Fludioxonil	39.0	40.3	43.5	44.6

of *transposa* and *vacuma* detected in the three cv. Moscato and the previously investigated vineyards was quite different. *Vacuma* strains were prevalent in all the cv. Moscato plots, while in the European and American vineyards examined so far, the percentage of *transposa* strains usually varied from 60 to approximately 90% and reached greatest proportions at harvest (Martinez *et al.*, 2005; Ma and Michailides, 2005; Kretschmer and Hahn 2008; Vaczy *et al.*, 2008; Esterio *et al.*, 2011; Samuel *et al.*, 2012). In previous studies *vacuma* isolates were detected on senescent floral parts and were only sporadically present or absent on ripe berries. On cv. Moscato, *transposa* strains were more abundant at harvest and during winter, as already observed by Martinez *et al.* (2005), but in all of the assessed phenological stages, the great majority of the *B. cinerea* strains associated with the sampled organs did not carry any TE. However, the

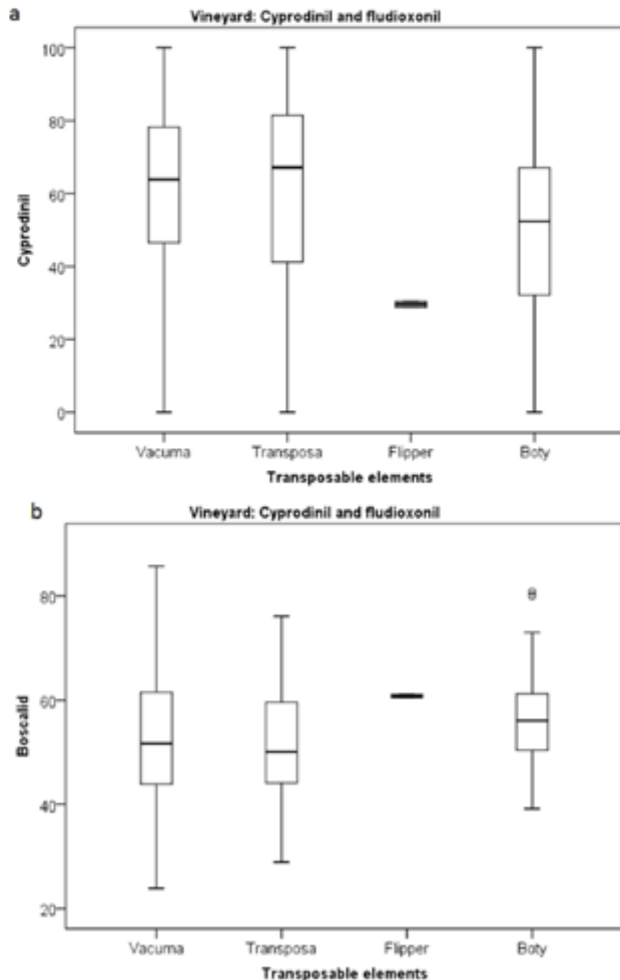

Figure 7. Box-plot distribution of growth inhibition percentage (GIPs) of the four *Botrytis cinerea* molecular types isolated in the vineyard treated with cyprodinil + fludioxonil relative to cyprodinil (a) and boscalid (b).Values more than 1.5 IQRs but less than three IQRs from either end of the box are labeled as outliers (O).

predominance of *vacuma* was more accentuated in the untreated and the cyprodinil + fludioxonil treated plots, while fenhexamid applications were associated with an analogous presence of *transposa* and *vacuma* individuals.

In other vineyards the prevalence of *transposa* isolates has been attributed mainly to their greater pathogenicity in comparison with the *vacuma* isolates, as assessed on inoculated berries of cv. Cabernet and Merlot collected at berry touch, veraison and harvest (Martinez *et al.*, 2005), but not confirmed on leaves (Martinez *et al.*, 2003). The lower virulence on berries and the more rapid growth on MA and PDA were attributed to the superior saprophytic ability of *vacuma* isolates which consequently preferentially colonize floral residues and continuously decrease from blossom onwards. Despite their paramount importance in shaping the *B. cinerea* populations, the phenotypic and pathogenic characteristics of the various TE types have generally not been further investigated. Only Samuel *et al.* (2012) did not confirm either the faster growth on artificial media or the lower virulence of *vacuma* strains. The wide collection of isolates assessed in the present study was particularly suited for evaluating the differences in virulence between TE types, and for the first time the pathogenicity of *Boty* and *Flipper*. Isolates obtained from cv. Moscato did not show any significant differences in pathogenicity associated with TEs on leaves or berries, indicating analogous ability of all molecular types to colonize grapevine vegetative or reproductive organs. The growth rate of cv. Moscato isolates was influenced by TEs only on the less complex medium, PDA, which supported more rapid development of *vacuma* strains.

Moreover, while abundant sclerotia, almost exclusively formed by *transposa* strains, were found on vine canes during dormancy (Martinez *et al.*, 2005), in Italian vineyards *B. cinerea* overwinters mainly as latent mycelium (Bisiach *et al.*, 1980). No sclerotia were detected in the cv. Moscato plots. Therefore, the more conspicuous presence of *vacuma* on cv. Moscato in comparison with other TE types, in particular *transposa,* can be related to its higher frequency among the overwintering strains and to its high level of virulence.

Boty isolates, a frequent component of the *B. cinerea* populations as already observed in Europe (Kretschmer and Hahn, 2008; Vaczy *et al.*, 2008; Samuel *et al.*, 2012) and America (Ma and Michailides, 2005; Esterio *et al.*, 2011), reached maximum occurrence at the beginning of berry touch. These isolates were obtained in analogous frequency from diseased and healthy berries collected in the three plots. *Flipper* individuals, less numerous than in Hungary (Vaczy *et al.*, 2008) and Greece (Samuel *et al.*, 2012), were isolated almost exclusively from asymptomatic organs at veraison and harvest.

Some phenotypic traits of *B. cinerea* communities were identified in the present study. The sclerotial/mycelial types and the average daily growth

rates varied according more to the fungicide treatment strategy than to the presence/absence of TEs. As already pointed out by Martinez *et al.* (2003), the majority of *B. cinerea* strains isolated from grapevine formed sclerotia on cultural media. Sclerotial isolates, belonging to all TE types, were significantly more frequent than the mycelial isolates in all the cv. Moscato sampled plots, but were particularly abundant in the vineyard treated with cyprodinil + fludioxonil, where a reduced percentage of individuals able to sporulate on cultural media was also detected. Since the ability to reproduce asexually strongly influences the dispersion of the pathogen, it is likely that the fitness of the isolates obtained from the vineyard treated with anilinopyrimidines and phenyl pirrol in mixture can be more severely affected in comparison with the other vineyards. Sclerotial isolates were particularly abundant at harvest and during dormancy. Since no sclerotia were detected in the sampled vineyards, sclerotial strains do not seem to have an ecological advantage over the mycelial strains during overwintering. Despite their abundance, sclerotial isolates do not possess any ecological advantages since no significant differences were found in the growth rates on MA and in virulence on leaves and berries, in comparison with the mycelial strains which, on the other hand, grew more rapidly on PDA.

Absence of fungicide treatments was associated with significantly more rapid hyphal development and reduced ability to cause disease symptoms on cv. Cabernet Sauvignon leaves in comparison with the application of botryticides. Since a faster growth rate on nutritive media suggests greater saprophytic potential (Pugh, 1980), the fungicide treatments apparently selected genotypes with reduced saprophytic ability and conversely with an enhanced virulence on grapevine vegetative organs. These results were not confirmed on berries, suggesting that infection of grapevine leaf tissues requires greater virulence. Berries represent a more favourable substrate for *B. cinerea* than leaves, due to the higher in pectin and sugar content found in fruit than in leaves.

The fungicide assays showed no differences in the growth inhibition between *B. cinerea* populations isolated from the differentially treated vineyards. In particular, an analogous sensitivity towards anilinopyrimidines, fludioxonil and fenhexamid characterized *B. cinerea* isolates obtained from treated and untreated vineyards. Only for vinclozolin a higher frequency of strains strongly sensitive to the fungicide was detected in the untreated in comparison with the treated vineyards. This was probably due to the intensive use of the fungicide in previous years. The application of fungicides against grey mould according to the IPM protocols adopted in Italy did not result in a strong selection pressure towards less susceptible individuals, at least in the investigated cases.

Botrytis cinerea isolates showed positive correlations between growth inhibition in the presence of vinclozolin and boscalid or fenhexamid. These fungicides are characterized by different mechanisms of action, i.e., respectively, osmotic signal transduction, mitochondrial respiration and sterol biosynthesis in membranes (Debieu *et al.,* 2001; Yamaguchi and Fujimura, 2005; Stammler *et al.,* 2007). The present results indicate that, in the monitored vineyards, the isolates characterized by poor growth inhibition by vinclozolin could at the same time be poorly controlled by boscalid and fenhexamid. Only a few isolates were characterized by unrestricted growth on the tested doses of two or more active substances: insensitivity to carbendazim was associated with poor growth inhibition by vinclozolin, fenhexamid or pyrimethanil. The distribution of GIPs induced by the assessed fungicides was not influenced by TEs, apart from *Flipper*: *B. cinerea* isolates harbouring only this TE showed reduced GIP distribution to cyprodinil and boscalid, suggesting that all *Flipper* strains are characterized by reduced sensitivity to cyprodonil and higher sensitivity to boscalid.

In conclusion, the differences in the *B. cinerea* populations associated with the sampled vineyards are not likely to be due to an isolation effect, since the cv. Moscato vineyards are almost adjacent to each other. The difference can therefore be attributed to the distinct treatment strategies. Thus, in northern Italy, the application of hydroxyanilide or anilinopyrimidine in mixture with phenyl pyrrole twice per year for at least 3 to 5 years has affected the phenotypic traits of the pathogen more than the TE distribution pattern of the *B. cinerea* populations. In each TE group, the selective pressure exerted by the fungicide applications induces decrease of the less competitive individuals from a pathogenicity point of view, particularly on leaves, resulting in a general reduction of the pathogen populations in the treated plots. The more favourable substrate represented by berries is likely to attenuate the selective pressure. The treat-

ment strategies based on 2-yearly applications of cyprodinil + fludioxonil or fehexamid did not have any significant effect on the overall response of *B. cinerea* populations to the fungicides, which is in agreement with the results obtained in Germany (Kretschmer and Hahn, 2008). Further investigations carried out in other vineyards are required in order to have better insights into *B. cinerea* populations associated with different grapevine cultivars, either untreated or treated with fungicides.

Dedication

This work is dedicated to Prof. Marco Bisiach.

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