European Journal of Plant Pathology **102:** 607–613, 1996.

Characterization and genetic analysis of field isolates of *Botryotinia fuckeliana* (*Botrytis cinerea*) resistant to dichlofluanid

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Accepted 22 March 1996

Key words: dichlofluanid, fungicides, genetics, resistance, grey mould

Abstract

Field isolates of *Botryotinia fuckeliana* were collected from naturally infected plants. Their responses to the multisite fungicide dichlofluanid in mycelium growth test fell into three phenotypic classes, characterized by the following EC_{50} (and MIC) values in $\mu g\ ml^{-1}$: sensitivity, 1–3 (6–10); low resistance, 3–10 (> 100); high resistance, 10–30 (> 100). The corresponding values obtained for these classes in a spore germination test were respectively: ≅ 0.05 (0.2), 0.05–0.1 (0.5), 0.5–1 (0.9–1.5). Resistant isolates were crossed with two sensitive and two resistant strains of appropriate mating type to determine the genetic basis of resistance. Distribution of resistance phenotypes in ascospore progeny indicated that a gene, named *Dic1*, was probably responsible for the low or high resistance of 14 mutants selectively collected from experimental plots of greenhouse-grown gerbera sprayed several times with dichlofluanid or tolyfluanid. A second gene, named *Dic2*, was probably responsible for the low resistance displayed by two isolates (from grapevine and from carnation) maintained in the laboratory collection. As a result of the investigation, the use of dichlofluanid in integrated management programmes against grey mould is discussed.

Introduction

Dichlofluanid, introduced in 1965 by Bayer AG (trade marks are 'Euparen' and 'Elvaron'), is a protective fungicide with a broad spectrum of activity against different pathogens, including *Botryotinia fuckeliana* (de Bary) Whetz. (teleomorph of *Botrytis cinerea* Pers.), which is the causal agent of grey mould disease on many plants throughout the world.

In recent years, the development of resistance of *B. fuckeliana* to benzimidazole fungicides (Dekker, 1977; Georgopoulos, 1979) and dicarboximide fungicides (Gullino and Garibaldi, 1986; Pommer and Lorenz, 1987) in several countries has caused much concern in grey mould control. Alternations or combinations with multisite chemicals such as dichlofluanid has been recommended to avoid the exclusive use of monosite fungicides and to cope with resistance.

There are several reports claiming dichlofluanid resistance in *B. fuckeliana* (Gjaerum and Munthe, 1985, 1987; Hunter et al., 1987; Malathrakis, 1989; Elena and Pappas, 1989; Rewal et al., 1991; Washington et al., 1992), but whether it really occurs has been doubted because of the broad variability in sensitivity to the fungicide observed among wild type isolates of the pathogen (Pappas and Elena, 1992). Moreover, Hunter et al. (1987) and Washington et al. (1992), contrary to findings of Leroux and Clerjeau (1985), found evidence of cross-resistance between dichlofluanid and dicarboximides.

Recently, in Italy, *B. fuckeliana* strains resistant to dichlofluanid were detected in low frequency in experimental plots of greenhouse grown gerbera plants as a result of 7–8 sprays with dichlofluanid or fungicide mixtures containing the analogous chemical tolyfluanid (Sansiviero et al., 1995). This paper reports investigations on the genetic basis of resistant phenotypes, aiming at clarifying the source of the pathogen's variability.

Materials and methods

Media

The following media were used (ingredients per l^{-1} distilled water): water agar (WA, 20 g agar), malt extract agar (MEA, 20 g Oxoid malt extract and 20 g agar), dextrose agar (DA, 10 g dextrose and 20 g agar), potato dextrose agar (PDA, infusion from 200 g peeled and sliced potatoes kept at 60 °C for 1 h, 20 g glucose and 20 g agar, adjusted at pH 6.5). Agar was technical grade (Oxoid N. 3).

Fungicides

Dichlofluanid (wettable powder containing 50% a.i., Euparen, Bayer) was suspended in sterile water; the dicarboximide fungicide vinclozolin (technical grade, supplied by BASF) and the benzimidazole fungicide benomyl (technical grade, supplied by Du Pont Conid) were dissolved in dimethylsulphoxide. The fungicides were added to autoclaved media that had cooled down to 45–50 °C. The concentration of dimethylsulphoxide did not exceed 1 μl ml^{-1}.

Isolates of Botryotinia fuckeliana

In this investigation, 55 *B. fuckeliana* isolates with different levels of sensitivity to dichlofluanid were used as a representative sample of numerous strains from gerbera plants. They derived from single conidia collected from naturally infected tissues. Resistant isolates were selected among conidia able to germinate on DA amended with 0.3 μg ml^{-1} dichlofluanid, which were transferred to MEA to obtain colonies (Sansiviero et al., 1995). *B. fuckeliana* isolates maintained in our laboratory collection (13 from grapevine and 7 from greenhouse crops) were screened for resistance; they had been collected in fields subject to intensive dichlofluanid applications.

Two strains from single ascospores (SAS56 and SAS405), of opposite mating type and sensitive to dichlofluanid, were used as reference strains in a first set of sexual crosses with resistant isolates; SAS56 was sensitive to benzimidazoles and dicarboximides (*MAT1-1 Mbc1S Daf1S*) and SAS405 was resistant to both groups of fungicides (*MAT1-2 Mbc1HR Daf1LR*) (Faretra et al., 1988b; Faretra and Pollastro, 1991). Genetic symbols and terminology for *B. fuckeliana* isolates have been described by Faretra and Grindle (1992). In particular, the *MAT1* gene is responsible of mating type, alleles of the *Mbc1* gene cause low (LR) or high (HR) resistance to benzimidazoles, and alleles of the *Daf1* gene cause low (LR) or high (HR) resistance to dicarboximides.

All isolates were maintained at –80 °C and transferred to MEA just before use.

Phenotypic characterization of B. fuckeliana *isolates*

Isolates were tested for mycelial growth on MEA and MEA amended with dichlofluanid (6 concentration levels, 1 to 100 μg ml^{-1}) to evaluate EC_{50} (Effective Concentration 50%) and MIC (Minimal Inhibitory Concentration). Three replicated 100-mm Petri dishes were inoculated with 4-mm plugs collected from the margin of actively growing colonies and kept at 21 ± 1 °C in the dark for 4 days. Two orthogonal diameters of colonies were then measured.

Five representative isolates for each phenotype were used in a germination test. Conidia were scraped from the surface of 7–10 day-old colonies grown on PDA and suspended in sterile water containing 0.05% Tween 20. Aliquots (15 μl) of conidial suspensions (10^6 conidia ml^{-1}) were spotted (200–250 conidia mm^{-2}) on DA and DA added with dichlofluanid (11 concentration levels, 0.05 to 3 μg ml^{-1}) and kept at 21 ± 1 °C for 24 h (control medium) or 48 h (dichlofluanid amended media). These conditions enhanced differences among phenotypes. Lactophenol-cotton blue was then added to stain conidia and to prevent further growth. A random sample of 100 conidia was observed at × 125 magnification and conidia which emitted germ tubes were counted in each of three replicated spots per each condition.

Individual isolates were also tested for resistance to benzimidazole and dicarboximide fungicides through a mycelial growth test on MEA, MEA amended with 5 or 30 μg ml^{-1} vinclozolin and MEA with 10 μg ml^{-1} benomyl (Faretra and Pollastro, 1991).

Derivation of meiotic progeny and genetic analysis

Dichlofluanid resistant isolates were crossed with the two sensitive reference strains, SAS56 and SAS405, to obtain apothecia and ascospores. Dichlofluanid resistant progeny of the cross between SAS405 and one of resistant field isolates (WS290) were back-crossed with both SAS56 and SAS405 to identify two strains of opposite mating type that were then used as resistant reference strains (SAR3188 and SAR3189) in a new set of sexual crosses (see below).

Matings were carried out as described by Faretra et al. (1988a).

Ascospores from individual apothecia of crosses in which reference strains were the sclerotial parent were spread at low density on WA, collected singly under a dissecting microscope, and subcultured on MEA. The colonies so obtained were tested as described by Faretra and Pollastro (1991) for growth on differential media: MEA, MEA with 6, 10 or 30 μg ml^{-1} dichlofluanid, and MEA containing 5 μg ml^{-1} vinclozolin. Resistant phenotypes were distinguished from sensitive ones on the basis of the appearance of colonies after 2–3 days incubation at 21 ± 1 °C.

Data obtained from single apothecia were statistically analysed for the segregation of phenotypic characters and the distribution of alleles by means of the χ^2 test.

Results

Data from the colony growth test on *B. fuckeliana* isolates from gerbera are outlined in Figure 1. Three distinct groups of phenotypes with different response to dichlofluanid, which were provisionally named Dic, were recognised: (i) 10 isolates were sensitive (DicS; EC_{50} = 1–3 μg ml^{-1} and MIC = 6–10 μg ml^{-1}, occasionally very limited growth up to 100 μg ml^{-1}); (ii) 25 isolates were low-resistant (DicLR; EC_{50} = 3–10 μg ml^{-1} and MIC > 100 μg ml^{-1}); (iii) 20 isolates were high-resistant (DicHR; EC_{50} = 10–30 μg ml^{-1} and MIC > 100 μg ml^{-1}) to dichlofluanid (Figure 2). Of the isolates maintained in collection, 16 were DicS and only 4 were DicLR.

The three groups of phenotypes could be discriminated also by the test on conidia germination (Figure 3). Characteristic responses were: DicS, sensitive ($EC_{50} \cong$ 0.05 μg ml^{-1} and MIC = 0.2 μg ml^{-1}); DicLR, low-resistant (EC_{50} = 0.05–0.1 μg ml^{-1} and MIC = 0.5 μg ml^{-1}); DicHR, high-resistant (EC_{50} = 0.5–1 μg ml^{-1} and MIC = 0.9–1.5 μg ml^{-1}).

In agreement with findings by Hunter et al. (1988) careful standardisation of the experimental conditions and of the type of inoculum was crucial for these tests. The colony growth assay required young inoculum collected at the margin of actively growing colonies; mycelial plugs from aged colonies of sensitive isolates often produced sectors even on MEA amended with 100 μg ml^{-1} dichlofluanid, but the mycelium of the sectors did not show any decreased sensitivity when further tested. The concentration of conidia was crucial in the germination assay; conidia plated at a density higher than that used in this work germinated and yielded normal germ tubes even on DA amended with 1.5 μg ml^{-1} dichlofluanid, irrespective of resistance phenotypes.

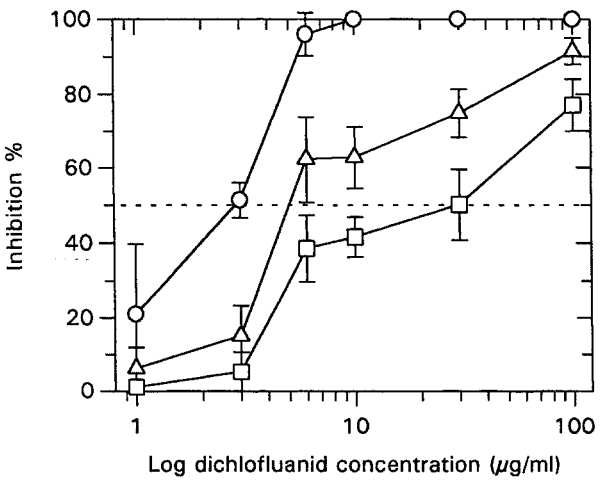

Figure 1. Percentage of inhibition of colony growth of *Botryotinia fuckeliana* field isolates on MEA amended with dichlofluanid. Figures are the average of 10 sensitive (○), 25 low resistant (△) and 20 high resistant (□) isolates; bars represent standard deviation.

Most dichlofluanid resistant isolates displayed also low-resistance to dicarboximides and high resistance to benzimidazoles, according to the phenotype classification reported by Faretra and Pollastro (1991; 1993a).

Eighteen representative dichlofluanid-resistant isolates, 8 DicLR and 10 DicHR, were crossed with reference strains SAS56 (*MAT1-1*) and SAS405 (*MAT1-2*). All isolates were fertile with the appropriate reference strain: 10 were MAT1-1 and 8 were MAT1-2. The progeny of fertile crosses was analysed. Most crosses yielded ascospore progeny in which sensitive and resistant phenotypes were in a statistically significant 1:1 ratio (Table 1). This indicated that resistant phenotypes of *B. fuckeliana* isolates were due to mutation in single major genes. Exceptions were the isolates WS180 and WS220 which did not transmit their phenotypic traits to the progeny so that all ascospores were sensitive. It is known that failure in character transmission to the progeny may occur in *B. fuckeliana* as a result of heterokaryosis of parental isolates (Faretra and Pollastro, 1991; 1993a; 1993b). Resistant isolates were then submitted to repeated sub-culturing; most resistant phenotypes were stable, but isolates WS180 and WS220 lost their resistance in about 50% of the transfers to a non-selective medium. This indicated that

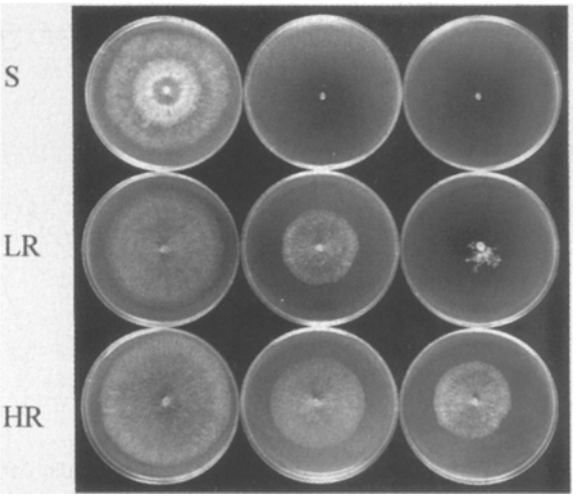

Figure 2. Resistance phenotypes (S = sensitive; LR = low resistant; HR = high resistant) grown on (from the left): MEA (control), MEA containing 10 or 30 μg ml^{-1} dichlofluanid.

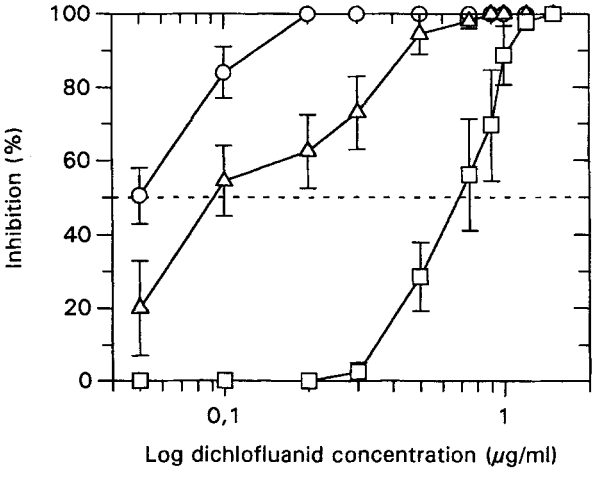

Figure 3. Percentage of inhibition of conidia germination of *Botryotinia fuckeliana* field isolates on DA amended with dichlofluanid. Figures are the average of 5 isolates for each phenotype: sensitive (○), low resistant (△) and high resistant (□); bars represent standard deviation.

these unstable isolates were probably heterokaryons, and they were no longer investigated.

The mating type of some DicHR ascospore progeny of the cross SAS405 × WS290 was determined and two monoascosporic strains, SAR3188 (*MAT1-1*) and SAR3189 (*MAT1-2*), were selected as putative resistant references to be used in a new set of crosses with isolates. Preliminarily, it was ascertained that DicS and DicR phenotypes were in similar proportions among ascospore progeny of crosses with sensitive strains, and that no sensitive phenotypes were produced by reciprocal crosses between the putative references (Table 2). This finding indicated that the

Table 1. Phenotypes and numbers in ascospore progeny of crosses between dichlofluanid-resistant field isolates and sensitive reference strains SAS56 or SAS405

Resistant isolates[1]		Ascospore progeny			χ^2 for a 1:1 segregation[2]
Ref. n.	Phenotype Dic	Total	Phenotypes DicS	DicR	
WS22	LR	223	120	103	1.30
WS76	LR	140	71	69	0.03
WS180	LR	135	135		na
WS220	LR	121	121		na
WS280	LR	104	57	47	0.96
WS283	LR	204	100	104	0.08
WS285	LR	201	91	110	1.80
WS286	LR	96	56	40	2.67
WS287	HR	94	54	40	2.09
WS290	HR	93	50	43	0.53
WS291	HR	188	90	98	0.34
WS292	HR	115	62	53	0.70
WS293	HR	94	43	51	0.68
WS294	HR	91	48	43	0.27
WS295	HR	94	55	39	2.72
WS296	HR	100	47	53	0.36
WS297	HR	89	44	45	0.01
WS298	HR	105	58	47	1.15

(1) Field isolates showed low resistance (LR) or high resistance (HR) to dichlofluanid.
(2) χ^2 value for 1 degree of freedom is 3.84 at P = 0.05 level of probability; na = not appropriate.

Table 2. Phenotypes and numbers in ascospore progeny of crosses involving two dichlofluanid highly resistant strains, SAR3188 and SAR3189, and two sensitive reference strains, SAS56 and SAS405[1]

Cross	Ascospore progeny			χ^2 for a 1:1 segregation[2]
	Total	Phenotypes		
		DicS	DicR	
SAR3189 × SAS405	100	47	53	0.36
SAR3188 × SAS56	116	64	52	1.24
SAR3188 × SAR3189	121		121	na
SAR3189 × SAR3188	130		130	na

[1] The monoascosporic strains were progeny of the cross SAS405 × WS290.
[2] χ^2 value for 1 degree of freedom is 3.84 at P = 0.05 level of probability; na = not appropriate.

two resistant strains, SAR3188 and SAR3189, were probably allelic.

Differential media did not permit a precise discrimination between DicLR and DicHR phenotypes in ascospore progeny of crosses between mutants and resistant reference strains; data referring to their segregations are therefore omitted. Recombinant DicS phenotypes were not detected among progeny of most crosses, but they were present in the offsprings of two DicLR isolates from the culture collection, WS22 and WS76 (Table 3). These isolates were then crossed, 109 ascospores were tested and all were found resistant to dichlofluanid. This finding indicated that the resistance phenotype of most DicLR or DicHR isolates was due to a mutation in the same gene or in closely linked genes. A second gene was probably responsible for the resistance displayed by the isolates WS22 and WS76.

Some of the crosses were appropriate to examine the reassortment of dichlofluanid resistance marker (Dic) and alleles of the *Mbc1* gene and the *Daf1* gene, which show a slight linkage and confer resistance to benzimidazole and dicarboximide fungicides, respectively (Faretra and Pollastro, 1991; 1993a). Dic and Mbc markers segregated independently in ascospore progeny and the ratio of recombinant to parental phenotypes was not significantly different from 1:1 (data not shown). Recombination between Dic and Daf markers was close to the expected 50% value for recombination of unlinked genes in ascospore progeny of mutants WS285, WS286 and WS287. However, there was a significantly higher frequency of parental phenotypes than recombinant phenotypes among ascospore progeny of mutants WS76, WS280 and WS283 (Table 4). This indicated that the dichlofluanid resistance

Table 3. Phenotypes and numbers in ascopore progeny of crosses between dichlofluanid-resistantfield isolates and dichlofluanid resistant strains SAR3188 or SAR3189

Resistant isolates		Ascospore progeny		
Ref. n.	Phenotype Dic	Total	Phenotypes	
			DicS	DicR
WS22	LR	127	13	114
WS76	LR	368	113	255
WS280	LR	130		130
WS283	LR	137		137
WS285	LR	126		126
WS286	LR	127		127
WS287	HR	132		132
WS290	HR	88		88
WS291	HR	111		111
WS292	HR	135		135
WS293	HR	116		116
WS294	HR	123		123
WS295	HR	118		118
WS296	HR	124		124
WS297	HR	114		114
WS298	HR	126		126

genes could be loosely linked to the *Daf1* gene in these isolates.

Conclusion

Dichlofluanid shows structural similarity to compounds like captan and folpet, one chlorine atom in the reactive trichloromehylthio moiety being replaced by a fluorine atom. The mode of action of this group of fungicides within a target organism is multisite; they react with thiol, SH and amino groups inducing formation of thiophosgene and hydrogen disulphide. Thus, they interfere with numerous metabolic steps and cause alteration of cellular structures (Luckens and Sisler, 1958; Siegel, 1971). Monogenic resistance to site-specific fungicides is a well known phenomenon in fungi, but single major genes responsible of stable resistance to multisite fungicides have been rarely reported (Grindle and Faretra, 1993).

This investigation has shown that, under strictly standardized experimental conditions, *B. fuckeliana* isolates may be grouped into three phenotypic classes (sensitivity, low resistance and high resistance) on the basis of their response to dichlofluanid. Resistance to dichlofluanid is encoded by at least two major genes which are both inherited in Mendelian fashion in

Table 4. Segregation of fungicide-resistance phenotypes among ascospore progeny of *Botryotinia fuckeliana*

Resistant isolates ref. n.	Parental phenotypes				Ascospore progeny						Recombination (%) between Dic and Daf markers	χ^2 for independent segregation of markers[2]
	Reference strain[1]		Resistant isolates		Total	Phenotypes (Dic-Daf)						
	Dic	Daf	Dic	Daf		S-S	S-LR	R-S	R-LR	Parental: recombinant		
WS76	S	LR	LR	S	140	33	30	55	22	85 : 55	39.3	6.4
WS280	S	S	LR	LR	94	34	23	14	23	57 : 37	39.4	9.1
WS283	S	S	LR	LR	203	57	39	41	66	123 : 80	39.4	4.3
WS285	S	S	LR	LR	205	42	39	58	66	108 : 97	47.3	0.6
WS286	S	S	LR	LR	96	32	24	20	20	52 : 44	45.8	0.7
WS287	S	S	HR	LR	114	29	35	22	28	57 : 57	50.0	0.0

[1] Reference strains were SAS405 (DicS DafLR) or SAS56 (DicS DafS).
[2] χ^2 values for 1:1 ratios of recombinant:parental phenotypes; χ^2 value for 1 degree of freedom is 3.84 at P = 0.05 level of probability.

meiotic progeny. One gene is probably responsible for the low or high resistance of 14 mutants selectively collected from greenhouse-grown gerbera plants sprayed several times with dichlofluanid or tolyfluanid. A second gene is probably responsible for the low resistance displayed by two isolates (WS22 from grapevine and WS76 from carnation) maintained in the laboratory collection. The two genes have been designated *Dic1* and *Dic2* in conformity with the system of genetic nomenclature proposed by Yoder et al. (1986), as detailed by Faretra and Grindle (1992). *Dic1S*, *Dic1LR* and *Dic1HR* have been assigned to the alleles of the *Dic1* gene coding for sensitivity, low resistance and high resistance, respectively. Likewise, *Dic2S* and *Dic2LR* have been assigned to the alleles of the *Dic2* gene coding for sensitivity and low resistance, respectively.

How resistance genes operate remains unknown. Resistance to a multisite fungicide like dichlofluanid cannot be caused by modification of the target sites, but it is likely to be due to alterate cell absorption or detoxification. Reaction with thiol groups has been postulated to act as a detoxifying mechanism when reactive thiols are not essential for growth (Lorbeer and Ellerbrock, 1976). Levels of glutathione higher than normal have been found in field isolates of *B. fuckeliana* resistant to captan; in response to the fungicide they also showed a faster and more intense synthesis of reduced glutathione than sensitive isolates (Barak and Edgington, 1984). Chlorothalonil forms conjugates with glutathione through glutathione S-transferase catalysed reactions (Ellner, 1993). These findings and the poor effectiveness of dichlofluanid against both conidia plated at high density and aged mycelium, would suggest that resistant genes might be involved in detoxifying mechanisms, and possibly in the regulation of thiol production.

Hunter et al. (1987) and Washington et al. (1992) found cross resistance between dichlofluanid and dicarboximide fungicides. Most of the dichlofluanid resistant isolates used in this investigation were also resistant to dicarboximides and benzimidazoles. Genetic analysis of their meiotic progeny, however, clearly showed the absence of any relationship among resistance to the different fungicides, since they are caused by different genes. It is therefore obvious that the presence of genotypes resistant to the different fungicides in a pathogen's population is the result of independent mutation events and of distinct selective forces operated by treatments.

Dichlofluanid is a powerful inhibitor of germination of conidia but shows limited activity against mycelium growth. In this investigation, the highest level of resistance (EC_{50} of resistant mutants/ EC_{50} of wild type isolates) observed in either germination or growth test was about 10. Thus, it is conceivable that the reduced sensitivity of resistant isolates may decrease the effectiveness of treatments with dichlofluanid in preventing infections by the mycelium. However, field rates of the fungicide should be still effective to prevent infections by conidia, since their germination is inhibited by concentrations as low as 1 $\mu g\ ml^{-1}$ even for high-resistant isolates. This might explain why in practice the presence of dichlofluanid-resistant isolates has been accounted for control failures only in few cases (Malathrakis, 1989). Spreading of resistant isolates in the field might have different relevance in decreasing the effectiveness of dichlofluanid under different conditions. Probably, a poor effectiveness may be expected in circumstances where, besides conidia,

mycelium living on plant debris plays an important role as inoculum for new infections. Therefore, integrated management programmes based on dichlofluanid should include appropriate cultural operations for keeping plants free from senescent leaves and plant debris, especially where dichlofluanid-resistant isolates are prevalent.

Acknowledgements

This investigation was supported by grants from Consiglio Nazionale delle Ricerche (project 'Genetics of *Botryotinia fuckeliana* (*Botrytis cinerea*)', which is part of the co-ordinate research programme 'Fungal genetics') and from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, 40% founds, project 'New crop protection strategies with low environmental risk').

We acknowledge gifts of the fungicides from BASF and Du Pont Conid.

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