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SBI-fungicides: fungicidal effectiveness and resistance in *Botrytis cinerea*

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Summary. *In vitro* fungitoxicity tests with 14 sterol biosynthesis inhibitors (SBIs) showed that the SBIs pyrifenox, flusilazol, propiconazole, triflumizole and fenpropimorph applied at the low concentration of 1 μg ml^{-1} inhibited the mycelial growth of wild-type and mutant strains of *Botrytis cinerea* that were resistant to the benzimidazoles, to the dicarboximides and to a mixture of benzimidazole+phenylcarbamate (carbendazim+diethofencarb). The SBIs tested exhibited higher effectiveness against the wild-type and mutant strains of *B. cinerea* compared with some widely used botryticides, such as the dicarboximides iprodione, procymidone and chlozolinate, the aromatic hydrocarbons quintozene, chloroneb and tolclofos-methyl, and the relatively new fungicides cyprodinil and fenhexamid. Only benomyl and fludioxonil presented higher effectiveness than the SBIs. *In planta* pot experiments with preventive applications of the commercial products Dorado 20 EC (pyrifenox), Punch 40 EC (flusilazol), Tilt 25 EC (propiconazole), Corbel 75 EC (fenpropimorph) and Trifmine 30 EC (triflumizole) showed that lesions of cucumber seedlings by all the above-mentioned strains of *B. cinerea* were completely inhibited at low SBI concentrations of 0.05–0.1 g a.i. l^{-1}. After chemical mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), only strains with low resistance (Rf 6–9, based on MIC values) to triadimefon were isolated at a mutation frequency of 3.9×10^{-5}. Cross-resistance studies with other SBIs showed that these triadimefon-resistant strains exhibited positive cross-resistance (Rf 2–10) to the other C-14 demethylase inhibitors (DMIs), but not to the morpholine fungicides fenpropimorph or tridemorph. Study of fitness of DMI-resistant strains showed that these mutation(s) were pleiotropic, with significant adverse effects on characteristics determining phytopathogenic fitness such as rate of mycelial growth, sporulation, conidial germination and pathogenicity on cucumber seedlings. The results indicate that some SBI-fungicides are suitable for use in resistance management programmes against grey mould.

Key words: grey mould, botryticides, ergosterol biosynthesis inhibitors, DMIs, morpholines.

Introduction

Grey mould caused by *Botrytis cinerea* is a serious disease on a wide range of crops with worldwide economic importance (Jarvis, 1980). Control of the fungus is based mainly on frequent applications of fungicides, but serious disease problems have resulted from the extensive appearance of resistant isolates. In the last three decades, chemical control of the pathogen has been severely restricted by the development of resistance to the intensively used benzimidazoles and dicarboximides (Lorenz, 1988; Smith, 1988).

In Greece, isolates resistant to the benzimidazoles appeared in the early 1970s (Malathrakis, 1979). The replacement of the benzimidazoles with the dicarboximide fungicides was only a temporary solution to the problem because in 1981 dicarboximide-resistant strains were also reported (Panayotakou and Malathrakis, 1983). Strains exhibiting a double resistance to both the benzimidazoles and dicarboximides were then also detected in several countries (Gullino and Garibaldi, 1986; Moorman

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and Lease, 1992). The fungicidal mixture of carbendazim plus diethofencarb was introduced in 1984 in an attempt to exploit the 'negative correlated cross-resistance' between the *N*-phenylcarbamates and the benzimidazoles (Kato *et al*., 1984). However, the intensive commercial use of this mixture in several countries such as Greece, Israel, France and Spain, led in a few years to the appearance of strains of the pathogen resistant to the benzimidazoles and without sensitivity to the phenylcarbamates (Katan *et al*., 1989; Leroux and Gredt, 1989; Raposo *et al*., 1994; Laskaris *et al*., 1996).

During the last few years, botryticides have been developed which belong to the new classes of phenylpyrroles, anilinopyrimidines, phenylpyridinamines and hydroxyanilidines (Leroux, 1996; Rosslenbroich and Stuebler, 2000). However, these new compounds are also threatened by resistance development, as indicated by recent reports (Faretra and Pollastro, 1993; Foster and Staub, 1996; Hilber and Hilber-Bodmer, 1998; Leroux *et al*., 1999; Ziogas and Kalamarakis, 2001).

B. cinerea is a classical 'high-risk pathogen' from the view of resistance management. The high adaptability of this pathogen to changing environmental conditions emphasizes the need for alternative fungicides in antiresistance strategies. Sterol biosynthesis inhibitors (SBIs) are a large and important group of site-specific inhibitors with a broad spectrum against several fungal diseases, especially powdery mildews, rusts and scabs (Buchenauer, 1995; Pommer, 1995). Although these fungicides have a broad antifungal activity, only some triazoles and imidazoles have been tested *in vitro* against *B. cinerea* (Kapteyn *et al*., 1994; Stehmann *et al*., 1994; Stehmann and De Waard, 1995, 1996a, 1996b), and data about their effectiveness *in planta* are very limited (Stehmann and De Waard, 1996b).

The present work was carried out to study the effectiveness of SBIs *in vitro* and *in planta* against strains of *B. cinerea* sensitive and resistant to benzimidazoles, to dicarboximides, and to a benzimidazole (carbendazim) and phenylcarbamate (diethofencarb) mixture. In order to increase our knowledge regarding the possibility of development of practical resistance of *B. cinerea* to the SBIs, the inherent resistance risk was also evaluated.

Materials and methods

Fungal strains and culturing conditions

The wild-type strain wt-B_1 of *Botrytis cinerea* Pers. ex Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] isolated from a tomato crop in Greece was used to obtain SBI-resistant mutants. In addition, the mutant strains of the pathogen B/BEN-1, B/IPR-1 and B/SU-1, resistant to the benzimidazoles, the dicarboximides and a mixture of carbendazim+diethofencarb respectively, obtained from the collection of the Plant Pathology Laboratory of Agricultural University of Athens, were tested for fungitoxicity, cross-resistance and pathogenicity. All strains were grown on potato dextrose agar (PDA) in a controlled-climate cabinet at 22°C with 14-h day^{-1} illumination provided by fluorescent tubes and 70% RH. For long-term storage the strains were maintained in glass tubes on PDA at 10°C in the dark, and single-tip transfers were made once a month.

Fungicides

All *in vitro* tested fungicides were pure technical grade and were kindly provided by their manufacturers. The SBIs used were: *(a)* C-14 demethylase inhibitors (DMIs) triforine, bitertanol, flusilazole, flutriafol, propiconazole, triadimefon, triadimenol, imazalil, clotrimazole, triflumizole, fenarimol and pyrifenox and *(b)* the morpholines fenpropimorph and tridemorph. The benzimidazole benomyl, the aromatic hydrocarbons quintozene (PCNB), chloroneb and tolclofos-methyl, the dicarboximides iprodione, procymidone and chlozolinate, a mixture of carbendazim and diethofencarb, the phenylpyrrole fludioxonil, the anilinopyrimidine cyprodinil and the hydroxyanilidine fenhexamid were also tested. Stock solutions of the fungicides were made in ethanol, with the exception of benomyl and fenhexamid, which were made in methanol and in isopropyl-alcohol respectively.

In the pathogenicity tests, aqueous suspensions of the commercial products 'Dorado' 20 EC (200 g l^{-1} pyrifenox), 'Punch' 40 EC (400 g l^{-1} flusilazol), 'Corbel' 75 EC (750 g l^{-1} fenpropimorph), 'Trifmine' 30 EC (300 g l^{-1} triflumizole), 'Tilt' 25 EC (250 g l^{-1} propiconazole), 'Rovral' 50 WP (500 g kg^{-1} iprodione), 'Benlate' 50 WP (500 g kg^{-1} benomyl) and 'Sumico' 25+25 WP (250 g kg^{-1} carbendazim and 250 g kg^{-1} diethofencarb) were used.

All fungicide concentrations are expressed as μg active ingredient per ml.

Mutation induction

Conidial suspensions (10^6 conidia ml^{-1}) of the wild-type strain (wt-B_1) of *B. cinerea* in water were obtained from 8–10-day-old slant cultures. They were agitated on a rotary shaker at 22ºC and 100 rev min^{-1} with 10 μg ml^{-1} *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) (Ziogas and Girgis, 1993) for 4 h in darkness and then washed twice with sterile distilled water. The mutagenic treatment resulted in 95% lethality. Conidia were resuspended in water, plated on PDA medium containing 100 μg ml^{-1} triadimefon and incubated at 22ºC for 20 days, to enable resistant colonies to appear. The selected resistant strains were maintained on PDA agar slants containing 25 μg ml^{-1} triadimefon, the minimal inhibitory concentration (MIC) for the wild-type strain.

***In vitro* fungitoxicity tests**

Fungicide sensitivity of the wild type and mutant strains of *B. cinerea in vitro* was assessed by measuring radial growth on PDA plates containing a range of concentrations of each fungicide. The experiments were conducted in 9-cm Petri dishes inoculated with 2-mm water-agar (WA) plugs on which conidia had been allowed to germinate. Agar plugs from cultures of *B. cinerea* were placed with the surface mycelium in direct contact with the medium. The effect of the fungicide on growth was determined by measuring the diameters of the mycelial colonies after incubation for 4–5 days at 22ºC in the dark. The fungicides were added aseptically to sterilized growth medium from stock solutions prior to inoculation. In all cases the final amount of solvent never exceeded 1% (v:v) in treated and control samples. At least six concentrations with three replicates for each fungicide were used to obtain the respective fungitoxicity curves. Concentrations causing a 50% reduction in the growth rate (EC_{50}) were determined using the dose response curves after probit analysis. The resistance level (resistance factor, Rf) was the ratio of the EC_{50} or MIC for a resistant strain to the EC_{50} or MIC for the parent sensitive strain.

Characteristics of resistant strains

Mutants of *B. cinerea* were tested for mycelial growth, sporulation and spore germination on PDA medium. Three 2-mm mycelial WA-plugs for each mutant were transferred to the centres of PDA plates to measure radial growth. After incubation at 22ºC in the dark, the colony diameter of each strain was measured at 24-h intervals. To determine conidial production in the absence of fungicides, PDA-plates were inoculated with a conidial suspension (10^6 conidia ml^{-1}) and were incubated for 8–10 days in a controlled climate chamber with a 14-h day^{-1}. The total mycelial mass produced in each dish was transferred to a 250-ml Erlenmeyer flask with 20-ml deionised water. The flasks were agitated vigorously and the concentration of the conidia in the resulting spore suspension, after filtration through cheesecloth, was measured with a Neubauer haemocytometer and expressed as number of conidia per cm^2 of plate culture. Spore germination was determined after 6-h incubation on PDA medium in the dark. Radial growth, sporulation and spore germination measurements were subjected to analysis of variance using Dunnett's multiple range test at P=0.05.

Pathogenicity tests

Pathogenicity and fungicide-resistance of various mutant strains of *B. cinerea* on plants were tested on cucumber seedlings (*Cucumis sativus* L. cv. Telegraph) according to the method described by Kato *et al*. (1984) with minor modifications (Ziogas and Girgis, 1993). Cucumber plant seedlings (four seedlings per 17-cm pot, two pots per treatment) grown in plastic pots for 8–10 days and at the cotyledon stage were used. The test fungicides in aqueous suspensions were sprayed to run-off at the desired doses with a hand-sprayer 5 h before inoculation. Control plants were sprayed with deionised water. The centre of each cotyledon was punctured with a needle and a 2-mm mycelial plug from the margin of a young colony on PDA medium was placed on the wound. The inoculated plants were incubated in a moist chamber at 22ºC for 3–5 days in the dark and the infection was scored by measuring the lesion on each cotyledon. Disease development was scored according to the following scale: 0, no infection; 0.5, infection only under the inoculum plug; 1, infection of less than 20% of cotyledon surface; 2, infection of 20–50% and 4, infection of more than 50%.

Statistical analysis

Analyses were made with the Statistical Analysis System (JMP, SAS Institute, Inc., Cary, NC, USA). The growth rate and the EC_{50} of each strain and fungicide were calculated from the data subjected to probit analysis. Dunnett's multiple range test was used to assess differences between mycelial growth, sporulation, spore germination and the pathogenicity ratings of the strains.

Results and discussion

Effectiveness of SBIs against *B. cinerea*

In vitro *fungitoxicity of SBIs against resistant to benzimidazoles, benzimidazoles+phenylcarbamates and dicarboximides*

The *in vitro* sensitivity of the wt-B_1 strain of *B. cinerea* to 14 SBIs and to representative botryticides from the fungicide groups of the benzimidazoles, aromatic hydrocarbons, dicarboximides, benzimidazoles+phenylcarbamates, phenylpyrroles, anilinopyrimidines and hydroxyanilidines is shown in Table 1. The wild-type strain of *B. cinerea* was highly sensitive to pyrifenox, triflumizole, flusilazole, propiconazole and fenpropimorph. The EC_{50s} and MICs of these SBIs were 0.03–0.18 and 1 μg ml^{-1} respectively. In addition, the above SBIs compared with the aromatic hydrocarbons quintozene, chloroneb and tolclofos-methyl, the dicarboximides iprodione, procymidone and chlozolinate, the anilinopyrimidine cyprodinil and the hydroxyanilidine fenhexamid exhibited higher effectiveness against the wild-type strain of *B. cinerea*. The fungitoxicity of the triazoles flutriafol, bitertanol and triadimenol, the imidazoles imazalil and clotrimazole, the pyrimidine fenarimol and the morpholine tridemorph, based on MIC values, were 2.5–10 times lower. However, this second group of SBIs, when compared with the aromatic hydrocarbons, dicarboximides, anilinopyrimidines and hydroxyanilidines tested, were equally effective against the wild-type strain of *B. cinerea*. Triadimefon and triforine were the least active SBI-fungicides tested. A high fungitoxicity against *B. cinerea* has also been observed with other triazoles, such as tebuconazole, itraconazole, cyproconazole and etaconazole (Stehmann and De Waard, 1995, 1996a, 1996b) and with the imidazole prochloraz (Kapteyn *et al*., 1994).

In vitro *fungitoxicity of SBIs against strains resistant to benzimidazole, benzimidazole + phenylcarbamate and dicarboximide*

As shown in Table 2, the laboratory mutant strains of *B. cinerea* B/BEN-1, B/IPR-1 and B/SU-1 were highly resistant respectively, to benomyl, iprodione and the mixture of carbendazim + diethofencarb, which were used for their selection.

Fungitoxicity tests with the SBIs pyrifenox, flusilazole, triflumizole, propiconazole, fenpropimorph, and imazalil against representative mutant strains of *B. cinerea* showed that the mutations for resistance to the benzimidazoles, dicarboximides and the mixture of benzimidazoles and phenylcarbamates did not affect the sensitivity of the strains to the SBIs (Table 2).

In planta *effectiveness of SBIs against strains sensitive and resistant to the benzimidazoles, the benzimidazoles + phenylcarbamates and the dicarboximides*

Pathogenicity and fungitoxicity studies with the resistant strains *in planta* showed that their resistance was also expressed when they were evaluated for infection ability on cucumber cotyledons preventively treated with the respective fungicides (Table 3). When cotyledons treated with benomyl or carbendazim+diethofencarb and iprodione where infected with the B/BEN-1, B/SU-1 and B/IPR-1 strains respectively, the infection rate was higher than that in untreated cotyledons. Apparently, such a type of benzimidazole-resistant strains quickly becomes dominant in the pathogen population in the field because it is as aggressive and virulent as sensitive strains (Georgopoulos, 1987; Nicot and Baile, 1996). In addition, the selection pressure caused by dicarboximide applications may increase the frequency even of strains with lower infection ability, such as B/IPR-1 (Table 3). Monitoring studies in Greece have shown that infections caused by strains resistant to dicarboximides or to benzimidazoles+dicarboximides are not common at the beginning of each new crop cycle, but their frequency increases with the seasonal fungicide application given (Pappas, 1997).

Unlike the above botryticides, the commercial SBIs Corbel 75 EC (fenpropimorph), Dorado 20 EC (pyrifenox), Punch 40 EC (flusilazol), Trifmine 30 EC (triflumizole) and Tilt 25 EC (propiconazole)

Table 1. EC_{50} and MIC of different fungicides for inhibition of mycelial growth of the wild-type strain wt-B_1 of *Botrytis cinerea*.

Fungicide	EC_{50}[a] (μg ml^{-1}) (mean±SE[b])	MIC[c] (μg ml^{-1})
Sterol Biosynthesis Inhibitors		
Triazoles		
Flusilazole	0.13±0.02	1
Propiconazole	0.14±0.06	1
Flutriafol	1.20±0.32	10
Bitertanol	1.56±0.25	10
Triadimenol	2.50±0.19	10
Triadimefon	5.90±0.67	25
Imidazoles		
Triflumizole	0.15±0.03	1
Imazalil	0.25±0.02	2.5
Clotrimazole	0.60±0.04	5
Pyrimidines		
Fenarimol	1.37±0.02	10
Pyridines		
Pyrifenox	0.03±0.001	1
Piperazines		
Triforine	1.68±0.23	25
Morpholines		
Fenpropimorph	0.18±0.015	1
Tridemorph	0.20±0.033	5
Benzimidazoles		
Benomyl	0.04±0.005	0.1
Aromatic hydrocarbons		
PCNB (quintozene)	0.25±0.023	10
Chloroneb	0.65±0.12	5
Tolclofos-methyl	0.10±0.036	5
Benzimidazoles + *N*-phenylcarbamate		
Carbendazim + Diethofencarb	0.18±0.027	0.5
Dicarboximides		
Iprodione	0.25±0.065	2.5
Procymidone	2.50±0.19	10
Chlozolinate	5.00±1.62	50
Phenylpyrroles		
Fludioxonil	0.005±0.0003	0.05
Anilinopyrimidines		
Cyprodinil	0.05±0.008	10
Hydroxyanilidines		
Fenhexamid	0.19±0.06	2.5

[a] Effective concentration causing 50% reduction in growth rate.
[b] Pooled standard error; three replicates.
[c] Minimal inhibitory concentration.

Table 2. Sensitivity of benzimidazole (mutant strain B/BEN-1), dicarboximide (mutant strain B/IPR-1) and benzimidazole-phenylcarbamate (mutant strain B/SU-1) resistant mutant strains of *Botrytis cinerea* to six SBIs.

Fungicide	Resistance factor[a] based on					
	EC50[b] (mean±SE[c])			MIC[d]		
	B/BEN-1	B/IPR-1	B/SU-1	B/BEN-1	B/IPR-1	B/SU-1
Benomyl	100.0±12.3	1.0±0.16	100.0±9.17	150	1	150
Carbendazim + Diethofencarb	1.3±0.38	1.0±0.28	59.0±3.66	1	1	40
Iprodione	0.7±0.02	400.0±16.7	0.9±0.04	0.8	>1000	0.8
Pyrifenox	1.9±0.63	2.3±0.21	2.3±0.14	1	1	1
Flusilazole	1.5±0.22	1.3±0.16	1.4±0.05	1	1	1
Triflumizole	1.4±0.12	1.3±0.01	1.4±0.28	1	1	1
Fenpropimorph	1.2±0.08	1.1±0.16	1.2±0.31	1	1	1
Propiconazole	1.4±0.26	1.3±0.29	1.3±0.03	1	1	1
Imazalil	1.2±0.31	1.2±0.06	1.1±0.19	1	1	1

[a] The resistance factor based on the EC_{50s} or the MICs is the ratio of the EC_{50} for the mutant to the EC_{50} for the wild-type, or the ratio of the MIC for the mutant to the MIC for the wild-type. The EC_{50} and the MIC of the wild-type (wt-B_1) strain are given in Table 1.
[b] Effective concentration causing 50% reduction in growth rate.
[c] Minimal inhibitory concentration.
[d] Pooled standard error; three replicates.

were highly effective against the wild-type and all the resistant strains. The fungicide concentrations completely inhibiting lesion development of the cotyledons from the wild-type and the mutant strains B/BEN-1, B/SU-1 and B/IPR-1 were similar to those of the botryticides Benlate 50 WP (benomyl), Sumico 50 WP (carbendazim+diethofencarb) and Rovral 50 WP (iprodione) against the wild-type strain.

In vivo experiments with tebuconazole, another triazole, also showed good performance of the fungicide on tomato plants and grape bunches. Its activity was only slightly lower than that of benomyl (Stehmann and De Waard, 1996b).

Study of inherent resistance risk for SBIs in *B. cinerea*

Selection of resistant strains

The inherent resistance risk of *B. cinerea* against SBIs was determined by various *in vitro* and *in planta* tests. Mutants resistant to triadimefon were isolated after chemical mutagenesis of the conidia of the wild-type strain of *B. cinerea* with a mutation frequency of 3.9×10^{-5}.

Laboratory mutants resistant to the SBIs are easy to generate in several fungi (De Waard, 1994; Hollomon, 1994). High mutation frequencies for resistance to SBIs have also been obtained in other pathogens: 9×10^{-5} for triadimenol resistance in *Nectria haematococca* var. *cucurbitae* (Kalamarakis *et al*., 1989); 2.4×10^{-5} for fenarimol resistance in the same organism (Kalamarakis *et al*., 1991); 1.6×10^{-5} for imazalil resistance in *Aspergillus nidulans*; and 2.5×10^{-5} for resistance to the same fungicide in *Penicillium cinerescens* (Van Tuyl, 1977). Similar mutation rates have been reported for resistance to the morpholine and piperidine fungicides in *Ustilago maydis* (Markoglou and Ziogas, 1999, 2000, 2001), *N. haematococca* (Demakopoulou *et al*., 1989; De Falandre *et al*., 1991) and *Aspergillus niger* (Engels *et al*., 1998).

As there were no noticeable differences in the growth of mutants on the control medium and on a triadimefon-containing medium, a sample of 10 such strains was chosen for further study. All triadimefon-resistant strains had low resistance to triadimefon, with Rf 3–7 based on EC_{50} and 6–9 based on MIC (Table 4). A dose-dependent decrease of growth was observed with wild-type and mutant strains (results not shown).

Cross resistance

To detect cross-resistance relationships, the sensitivity of representative triadimefon-resistant

Table 3. Effect of fungicides on lesion development following inoculation of cucumber seedlings with the sensitive, wild type strain (wt-B_1), and the resistant strains (B/BEN-1, B/SU-1, B/IPR-1, B/TDF-25, B/TDF-8) of *Botrytis cinerea*.

Fungicide concentration (μg a.i. ml^{-1})	Infection of cotyledons (% of control)					
	wt-B_1	B/BEN-1	B/SU-1	B/IPR-1	B/TDF-25	B/TDF-8
Control	100 (60 a[a])[b]	100 (64 a)[b]	100 (59 ab)[b]	100 (30 b)[b]	100 (25 c)[b]	100 (28 bc)[b]
Corbel 75 EC[c]						
25	6 b	8 b	5 bc	2 c	21 a	19 a
50	0.5 b	0 b	1 b	0 b	12 a	10 a
100	0 b	0 b	0 b	0 b	4 a	3 a
200	0 a	0 a	0 a	0 a	2 a	0 a
Dorado 50 EC[c]						
25	14 b	n.t.	n.t.	n.t.	36 a	33 a
50	5 b	3 b	1 bc	0 c	21 a	18 a
100	0 a	0 a	0 a	0 a	3 a	1 a
200	0 a	0 a	0 a	0 a	2 a	0 a
Punch 40 EC[c]						
25	25 b	n.t.	n.t.	n.t.	43 a	46 a
50	11 b	14 b	8 bc	5 c	28 a	25 a
100	2 b	3 b	1 b	0 b	11 a	9 a
200	0 a	0 a	0 a	0 a	2 a	0 a
Trifmine 30 EC[c]						
25	36 b	n.t.	n.t.	n.t.	55 a	53 a
50	15 b	12 b	13 b	7 c	41 a	38 a
100	9 b	8 b	6 b	2 c	15 a	13 a
200	2 a	1a b	0 b	0 b	4 a	3 a
Tilt 25 EC[c]						
25	32 b	28 b	30 bc	22 c	57 a	55 a
50	16 b	13 bc	15 b	9 c	30 a	27 a
100	4 b	6 b	0 c	0 c	14 a	11 a
200	0 a	0 a	0 a	0 a	3 a	2 a
Benlate 50 WP[c]						
50	0 b	86 a	84 a	0 b	0 b	0 b
Sumico 50 WP[c]						
100	0 b	0 b	60 a	0 b	0 b	0 b
Rovral 50 WP[c]						
200	0 c	0 c	9 b	79 a	0 c	0 c

[a] Within rows, values followed by the same letter do not differ significantly according to Dunnett's multiple range test (P=0.05).
[b] In parentheses the sum of indices in 16 cotyledons.
[c] Commercial formulations: Punch (flusilazole); Dorado (pyrifenox); Trifmine (triflumizole); Tilt (propiconazole); Corbel (fenpropimorph); Benlate (benomyl); Sumico (carbendazim+diethofencarb); Rovral (iprodione).
n.t., not tested.

strains (B/TDF) to the other SBIs was compared with the wild-type strain. The EC_{50} and MIC values for each fungicide and strain are given in Table 4. The mutation(s) for resistance to triadimefon slightly reduced sensitivity (Rf 2–10 based on MIC values) to all other C-14 DMIs. No cross-resistance relationship was observed between triadimefon and the morpholines fenpropimorph and tridemorph, which act at subsequent steps of the sterol biosynthetic pathway (Ziogas *et al*., 1991; Köller, 1992). A positive cross resistance between tebuconazole and triadimenol was also observed in another study on field isolates of *B. cinerea* (Stehmann and De Waard, 1996a).

Table 4. Reduced sensitivity to sterol biosynthesis inhibitors of four representative triadimefon-resistant mutant strains (B/TDF-25, B/TDF-39, B/TDF-8, B/TDF-6) of *Botrytis cinerea*.

Fungicide	Resistance factor[a] based on							
	EC50[b] (mean ± SE[c])				MIC[d]			
	B/TDF-25	B/TDF-39	B/TDF-8	B/TDF-6	B/TDF-25	B/TDF-39	B/TDF-8	B/TDF-6
Triazoles								
Triadimefon	6.8±0.33	4.3±1.17	3.9±0.43	3.8±0.47	9	8	8	6
Triadimenol	4.2±1.17	2.6±1.09	3.6±1.22	1.2±0.39	10	10	7.5	7.5
Propiconazole	3.6±1.26	2.3±0.16	2.6±1.09	3.3±1.44	10	10	10	5
Bitertanol	2.5±0.29	1.7±0.09	1.4±0.21	2.1±1.17	7.5	5	5	5
Flutriafol	8.3±1.67	5.3±1.57	5.6±0.36	4.7±1.29	10	10	7.5	7.5
Flusilazole	3.2±0.12	2.4±0.27	1.3±0.07	1.2±0.33	10	5	5	5
Imidazoles								
Clotrimazole	4.2±0.18	2.8±0.03	3.2±1.05	1.6±0.73	10	10	10	5
Imazalil	2.7±0.06	2.4±0.18	1.6±0.27	1.3±0.02	4	4	4	3
Triflumizole	3.3±0.34	2.7±1.17	3.0±1.84	3.2±1.05	10	10	7.5	7.5
Pyrimidines								
Fenarimol	6.3±1.86	5.1±0.23	5.4±1.73	4.8±0.56	5	5	5	2.5
Pyridines								
Pyrifenox	5.7±1.24	5.2±1.04	3.8±1.46	3.1±0.38	5	2.5	2.5	2.5
Piperazines								
Triforine	3.1±0.22	3.7±1.68	2.1±0.87	2.6±1.62	5	4	4	2
Morpholines								
Fenpropimorph	2.6±0.83	2.2±0.03	1.5±0.44	1.4±0.17	1	1	1	1
Tridemorph	3.6±1.11	2.6±0.31	2.4±1.38	2.8±1.21	1	1	1	1

[a] The resistance factor based on the EC_{50s} or the MICs is the ratio of the EC_{50} for the mutant to the EC_{50} for the wild-type, or the ratio of the MIC for the mutant to the MIC for the wild-type. The EC_{50} and the MIC of the wild-type (wt-B_1) strain are given in Table 1.
[b] Effective concentration causing 50% reduction in growth rate.
[c] Pooled standard error; four replicates.
[d] Minimal inhibitory concentration.

Table 5. Comparison of mutants (B/TDF-6, B/TDF-8, B/TDF-39, B/TDF-25) of *Botrytis cinerea* resistant to DMI-fungicides with their parental wild-type strain (wt-B_1) in respect of some saprophytic fitness parameters on agar medium.

Strain	Radial growth[a]	Sporulation[b]	Spore germination[c]
wt-B_1	52 a[d]	8.2 a	87.4 a
B/TDF-6	25 b	5.4 b	40.9 b
B/TDF-8	20 b	4.2 b	38.5 b
B/TDF-39	11 c	3.5 c	22.4 c
B/TDF-25	10 c	2.9 c	19.7 c

[a] Mean colony diameter (mm) after 4 days of incubation (n=3)
[b] Mean number ($\times 10^6$) of conidia per cm^2 of colony after 10 days' incubation (n=3).
[c] Percent of germinated conidia after 6-h incubation (n=100).
[d] Within columns, values followed by the same letter do not differ significantly according to Dunnett's multiple range test (P=0.05).

Growth rate, sporulation and spore germination measurements

A comparison of some fitness-determining parameters between representative triadimefon-resistant strains and the parent strain, showed that triadimefon resistance was accompanied by significant reductions in the growth rate (50–80%), sporulation (35–65%) and spore germination (53–77%) (Table 5). Lower growth rate, sporulation and spore germination were also observed in the B/IPR-1 strain that was highly resistant to the dicarboximide iprodione. By contrast, a reduced sensitivity to the benzimidazoles and phenylcarbamates was not accompanied by a lowering of saprophytic fitness characteristics (results not shown).

The pleiotropic effect of mutations for resistance to SBIs are known from many studies on other pathogens (Kalamarakis *et al*., 1991; De Waard, 1994; Hollomon, 1994; Markoglou and Ziogas, 1999, 2000).

Pathogenicity and fungicide resistance of SBIs-mutant strains on plants

The infection ability of mutant strains is an important factor when assessing the risk for resistance development. A study of the phytopathogenic fitness of triadimefon-resistant strains of *B. cinerea* showed that the mutation(s) for resistance to SBIs appeared to have significant adverse effects on the virulence of mutant strains on cucumber seedlings. A reduction of approximately 55% in lesion development from the B/TDF-mutant strains was observed in untreated cotyledons, compared with that of the wild-type strain (Table 3). Furthermore, the low *in vitro* resistance of B/TDF-resistant strains to SBIs was difficult to evaluate *in planta*. The commercial SBIs tested were almost equally effective against both wild-type and SBIs-mutant strains of *B. cinerea*.

Conclusion

The ability of *Botrytis cinerea* to adapt rapidly to specific fungicides is a serious problem for effective chemical control of grey mould in many crops all over the world. When applying fungicides against grey mould of greenhouse crops and transported and stored fruits, flowers and vegetables, an important rule to avoid the development of pathogen resistance is to alternate fungicides with different modes of action. SBIs from all chemical subclasses are effective inhibitors of grey mould. SBIs may be a possible alternative fungicide group in *B. cinerea* control programs, due to the absence of cross-resistance between SBIs and the other botryticides. They present a number of advantages over other fungicides including a broad spectrum of fungicidal activity and a relatively low resistance risk, as shown by the present study and other reports in the literature (De Waard, 1994; Hollomon, 1994).

Acknowledgements

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