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Identification of a novel point mutation in the β-tubulin gene of *Botrytis cinerea* and detection of benzimidazole resistance by a diagnostic PCR-RFLP assay

Basil N Ziogas · Dimitra Nikou · Anastasios N Markoglou · Anastasios A Malandrakis · John Vontas

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Abstract The molecular basis of resistance to benzimidazole fungicides with laboratory and field mutant isolates of *Botrytis cinerea* was investigated. After chemical mutagenesis with *N*-methyl-*N*-nitrosogouanidine (NMNG) two different benzimidazole-resistant phenotypes were isolated on media containing carbendazim or a mixture of carbendazim and diethofencarb. The mutant isolates from the fungicide-mixture-containing medium were moderately resistant to carbendazim with wild-type tolerance to diethofencarb while mutant isolates from carbendazim-containing medium were highly resistant to carbendazim but sensitive to diethofencarb. The studied field isolates were highly resistant to benzimidazoles and sensitive to diethofencarb. Study of fitness characteristics of benzimidazole highly-resistant isolates showed that the resistance mutation(s) had no apparent effect on fitness-determining parameters. Contrary to this, the moderately benzimidazole-resistant strains, with no increased diethofencarb sensitivity, had a significant reduction in certain ecological fitness-determining characteristics. Analysis of the sequence of the β-tubulin gene revealed two amino acid replacements in the highly benzimidazole-resistant mutants compared to that of the wild-type parent strain. One was the glutamic acid (GAG) to alanine (GCG) change at position 198 (E198A), identified in both laboratory and field highly benzimidazole-resistant isolates, a mutation previously implicated in benzimidazole resistance. The second was a novel benzimidazole resistance mutation of glutamic acid (GAG) to glycine (GGG) substitution at the same position 198 (E198G), identified in a highly benzimidazole-resistant laboratory mutant strain. Molecular analysis of the moderately benzimidazole-resistant strains revealed no mutations at the β-tubulin gene. A novel diagnostic PCR-RFLP assay utilising a *Bsa*I restriction site present in the benzimidazole-sensitive (E198) but absent in both resistant genotypes (E198G and E198A) was developed for the detection of both amino acid replacements at the β-tubulin gene.

Keywords β-tubulin mutations · Molecular diagnostic assay · Diethofencarb sensitivity · Monitoring resistance

B. N Ziogas (✉) · D. Nikou · A. N Markoglou · A. A Malandrakis · J. Vontas
Laboratory of Pesticide Science,
Agricultural University of Athens,
Votanikos 118 55 Athens, Greece
e-mail: ziv@aua.gr

Introduction

Grey mould caused by *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*) is one of the most serious diseases on a wide range of crops with world-wide

economic importance. The main measure to reduce the incidence of grey mould in most crops, especially under greenhouse conditions, is the frequent application of fungicides. However, chemical control of the pathogen has suffered heavily from the development of resistance to the intensively used site-specific fungicides such as benzimidazoles and dicarboximides (Lorenz 1988; Smith 1988) and the major uses of benzimidazoles have been lost. However there are situations where strategies have been used successfully to delay resistance problems (Brent and Hollomon 1998).

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 since in 1981 dicarboximide-resistant strains were also reported (Panayotakou and Malathrakis 1983). Strains exhibiting a double resistance to both the benzimidazoles and dicarboximides emerged shortly afterwards in several countries (Gullino and Garibaldi 1986; Moorman and Lease 1992). A fungicidal mixture of carbendazim and diethofencarb was introduced in 1984 in an attempt to exploit the 'negatively 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 within a few years to the selection of strains of the pathogen resistant to the benzimidazoles and without sensitivity to the phenylcarbamates (Faretra et al. 1989; Katan et al. 1989; Leroux and Gredt 1989; Raposo et al. 1994; Laskaris et al. 1996).

Various *B. cinerea* benzimidazole-resistant isolates have been found in several crops throughout the world (Stehmann and de Waard 1996). In most cases, resistance is characterised by its high persistence in the field long after the interruption of fungicide applications (Georgopoulos and Skylakakis 1986) and has been attributed to β-tubulin modifications. The well characterised amino acid substitutions E198A, E198V, E198K and F200Y were found responsible for this target site insensitivity in benzimidazole-resistant *B. cinerea* isolates (Yarden and Katan 1993; Leroux et al. 2002). Furthermore, to monitor this type of resistance, Luck and Gillings (1995) and recently Banno et al. (2008) developed polymerase chain reaction (PCR)-based diagnostic assays to detect the E198A resistance mutations in *B. cinerea* isolates.

In this study we analysed the benzimidazole target site β-tubulin sequences from benomyl-resistant laboratory mutants and field isolates. We report the detection of a novel resistant mutation at amino acid 198 (E198G) and the adaptation of the previously developed polymerase chain reaction-based diagnostic assay (PCR-RFLP) in order to differentiate both resistant mutations, E198A and E198G, from the wild-type allele.

Materials and methods

Fungal strains and culture conditions

Chemical mutagenesis was used to obtain carbendazim-resistant isolates from a strain of *B. cinerea* (DSM 877) with wild-type sensitivity to benzimidazoles, from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). Field isolates resistant to benzimidazoles from strawberry crops in Greece were also used in this study. All isolates were grown on potato dextrose agar (PDA) in a controlled climate cabinet at 22°C with a 14 h day^{-1} light and 70% relative humidity. For long-term storage the isolates were maintained in glass tubes on PDA at 10°C in the dark and single-tip transfers were made once a month.

Fungicides

The fungicides used were pure technical grade. Carbendazim and fenhexamid were kindly supplied by Bayer CropScience AG (Leverkusen, Germany), benomyl by Du Pont de Nemours and Co. (Wilmington, DE, USA), pyraclostrobin and boscalid by BASF AG (Limburgerhof, Germany), azoxystrobin, fludioxonil and cyprodinil by Syngenta Crop Protection AG (Basle, Switzerland), fluazinam by ISK Biosciences Ltd (Kent, UK), iprodione by Rhone Poulenc Agro, Lyon, France (now Bayer Crop Science), dienthofencarb and the wettable powder formulation 'Sumico' (carbendazim + diethofencarb) by Sumitomo Chemical Co. (Osaka, Japan). Stock solutions of fungicides were made in ethanol, with the exception of benomyl and fenhexamid which were dissolved in acetone and in isopropanol, respectively.

Mutation induction

Mutant strains resistant to carbendazim were obtained after chemical mutagenesis with 10 μg ml^{-1} N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and selection on PDA medium containing carbendazim (5 μg ml^{-1}) or carbendazim (5 μg ml^{-1}) plus diethofencarb (5 μg ml^{-1}) after incubation at 22°C for 15 days, to enable resistant colonies to appear. The selected resistant isolates were maintained on PDA agar slants containing 1 μg ml^{-1} carbendazim, the minimal inhibitory concentration (MIC) for the wild-type parent strain of *B. cinerea*.

In vitro fungitoxicity tests

The fungicide sensitivity of the wild-type and mutant strains was assessed by inoculating PDA plates with mycelial inoculum consisting of 2-mm plugs cut from water-agar (WA) medium on which conidia of *B. cinerea* had been allowed to germinate, after overnight incubation at 22°C. The fungicides were added aseptically to sterilised 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. The effect of the fungicide on growth was determined by measuring the diameter of mycelial colonies after incubation for 4 days at 22°C in the dark. The EC_{50} values were determined from dose-response curves after probit analysis. The ratio of EC_{50} for a resistant isolate to the EC_{50} for the parent sensitive strain gave an estimation of the resistance level (resistance factor, Rf).

Determination of saprophytic and parasitic fitness parameters

Mutants of *B. cinerea* were tested for mycelial growth rate, sporulation, sclerotial production and pathogenicity compared to the wild-type parent strain. Radial growth measurements were made after incubation at 22°C in the dark, by recording the colony diameter of each isolate at 24 h intervals. Conidial production in the absence of fungicides was assessed after inoculation of PDA plates with a conidial suspension (10^5 conidia per plate) and incubation for 10 days at 22°C with a 14 h day^{-1} light. Sclerotial production was determined after 20 days of incubation on PDA medium in the dark.

Pathogenicity of the wild-type and mutant isolates of *B. cinerea* was determined by examining symptom severity caused by each strain on cucumber seedlings (*Cucumis sativus*, cv. Telegraph) according to the method described previously by Ziogas and Girgis (1993). Cucumber seedlings grown in plastic pots for 8–10 days (four seedlings per 17-cm pot, two pots per treatment) were used at the cotyledon stage. 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 was placed on the wound. The inoculated plants were incubated in a moist chamber at 22°C in the dark for 3–5 days and the infection was scored by evaluating the lesion on each cotyledon. Disease development was evaluated according to the following indices: 0, no infection; 0.5, rot only under inoculum; 1, < 20% rot; 2, 21–50% rot and 4, rot on > 50% of cotyledon surface.

Extraction of DNA and RNA and cDNA synthesis

Fungal cultures were grown over a disc of sterile cellophane on PDA at 25°C for 3 days. Mycelium was peeled and ground in liquid nitrogen using a mortar and pestle. Total RNA was isolated using TRI reagent (Sigma) according to the manufacturer's instructions. The RNA was treated with DNase RQ1 (Promega) to remove any contaminating genomic DNA. First-strand cDNA was synthesised from total RNA using SuperScript II RNase H^- reverse transcriptase (Invitrogen) and an oligo(dT) adaptor primer [5′-GACTCGAGTCGATCGA $(-dT)_{17}$- 3′] as described in the manufacturer's protocol. Genomic DNA was extracted using TRI reagent according to the manufacturer's instructions.

Sequence analysis of β-tubulin gene from *B. cinerea* strains

The specific oligonucleotide primers bctubF (5′AGG TACCATGGATGCTGTCC 3′) and bctubR (5′AAA ATGGCAGAGCATGTC AA 3′) were designed based on the partial sequence of the *B. cinerea* β-tubulin (X73133). These primers were used to amplify a 751 bp partial gDNA (codons 109 to 418) in a PCR reaction containing 0.2 μM each of 1.5 mM $MgCl_2$, 0.5 mM dNTPs, and 1.25 units of HotStar Taq DNA

polymerase (Qiagen) in 20 mMTris-HCl and 50 mM KCl. Genomic DNA and cDNA from benzimidazole-sensitive and resistant isolates were used as templates. The PCR conditions were 95°C for 15 min and then thirty-five cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min with a final 10 min extension at 72°C. The PCR products of the expected size were ligated to pGEM-TEasy vector and at least ten independent clones from each strain were sequenced in both directions and analysed. Sequence data were analysed using Lasergene software (DNAstar, Madison, USA).

PCR-RFLP assay to detect benzimidazole resistance-associated mutations

Primers bcF (5′ GGCTACCTTCTCCGTCGTC 3′) and bctubR were used to amplify a 459 bp gDNA β-tubulin fragment from *B. cinerea* benzimidazole-sensitive and resistant strains. The PCR conditions were 95°C for 15 min and then thirty-five cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final 10 min extension at 72°C. The PCR products were purified using QiaQuick columns (Qiagen) and one fifth of the products was digested with the restriction enzyme *Bsa*I in manufacturer's reaction buffer, and electrophoresed on a 2.5% agarose gel.

Statistical analysis

Data analyses were made with the Statistical Analysis System (JMP, SAS Institute, Inc., Cary, NC, USA). The growth rate and the EC_{50} value for each isolate and fungicide were calculated from the data subjected to probit analysis. Dunnett's multiple range test was

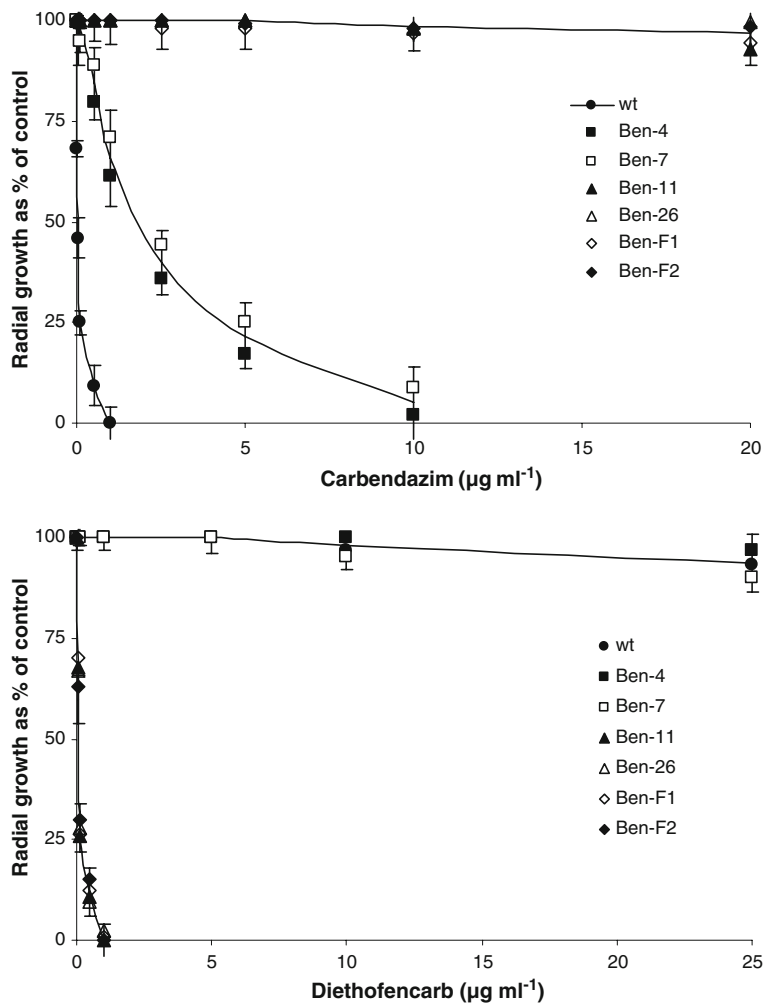

Fig. 1 Sensitivity of the wild-type (wt-B_1) and representative benzimidazole-resistant isolates of *Botrytis cinerea* to carbendazim and diethofencarb on PDA medium. Measurements were made after 4 days incubation

used to assess the differences between mycelial growth rates, sporulation, sclerotial production and pathogenicity ratings of isolates.

Results

Isolation and characterisation of benzimidazole-resistant mutants of *B. cinerea*

Mutant strains of *B. cinerea* resistant to carbendazim with or without wild-type tolerance to diethofencarb were isolated after chemical mutagenesis and selection on media containing carbendazim or carbendazim + diethofencarb, respectively. From the carbendazim-containing medium, 428 resistant colonies were obtained, indicating a mutation frequency of 1.2×10^{-5}. On the medium containing carbendazim plus diethofencarb, only four resistant isolates appeared, indicating a frequency of mutation to benzimidazole resistance of 1.1×10^{-7}, with no change in diethofencarb sensitivity. These mutants exhibited low to moderate resistance to carbendazim with a resistance factor ranging from 22 to 27. Results of the *in vitro* sensitivity assays with representative mutant strains and the wild-type parent strain of *B. cinerea* are shown in Fig. 1.

Study of some fitness-determining characteristics in the wild-type and carbendazim-resistant mutants from the diethofencarb-sensitive phenotypic class showed that the mutation(s) leading to benzimidazole resistance had no effect on mycelial growth and other fitness-determining parameters such as sporulation, sclerotial production and pathogenicity. On the contrary, comparison between carbendazim-resistant mutants with no increased diethofencarb sensitivity and the parent wild-type strain of *B. cinerea* showed that most of these fitness parameters were significantly reduced in these mutant isolates (Table 1).

Cross-resistance

The cross-resistance patterns of carbendazim with other benzimidazoles and with fungicides affecting

Table 1 Comparison of *Botrytis cinerea* isolates resistant to carbendazim with their parental wild-type strain with respect to some fitness parameters

Strains	Resistance factor[a] based on EC_{50}[b] (mean ± SE[c])	Radial growth[d]	Sporulation[e]	Sclerotial production[f]	Pathogenicity [g]
wt (DSM 877)		51a[h]	8.8a[h]	97.2a[h]	100a[h]
Laboratory mutants					
Ben-11 (BrDs)	235 (±5)	48a	9.2a	89.3ab	98.4a
Ben-23 (BrDs)	237 (±8)	52a	9.0a	93.8a	100a
Ben-26 (BrDs)	240 (±3)	50a	8.7a	95.7a	97.8a
Ben-4 (BrDr)	27 (±3)	38b	5.9b	69.4c	45.3b
Ben-7 (BrDr)	23 (±2)	29c	3.4c	53.6c	37.2c
Ben-12 (BrDr)	26 (±4)	22c	3.8c	48.5d	29.6cd
Ben-27 (BrDr)	22 (±3)	34b	4.6bc	50.2cd	50.3b
Field strains					
Ben-F1 (BrDs)	228 (±6)	51a	8.6a	98.3a	99.7a
Ben-F2 (BrDs)	254 (±4)	49a	9.1a	94.7a	100a

[a] The ratio of EC_{50} for mutant : EC_{50} for wild-type

[b] Effective concentration causing 50% reduction in growth rate after 4 days incubation at 22°C (n=3)

[c] Pooled standard error; three replications

[d] Mean colony diam (mm) measurements after 4 days incubation (n=3)

[e] Mean number (x 10^6) of conidia cm^{-2} of colony after 10 days incubation (n=3)

[f] Mean dry weight of sclerotia (mg) per plate after 20 days incubation (n=3)

[g] Pathogenicity as % of the wild-type. The sum of indices of eight cucumber seedlings for the wild-type was 60 (n=16)

[h] Within columns, values followed by the same letter do not differ significantly according to Dunnett's multiple range test (P=0.05)

other cellular pathways are shown in Table 2. The mutation(s) for resistance to carbendazim reduced the sensitivity of mutant strains to benomyl (Rf: 16-225, based on EC_{50} values), but not to the phenylpyrrole fludioxonil, the dicarboximide iprodione, the hydroxyanilide fenhexamid, the anilinopyrimidine cyprodinil, the carboxamide boscalid, the strobilurin-type fungicides azoxystrobin and pyraclostrobin and to the phenylpyridinamine fluazinam.

Amino acid sequence of the β-tubulin gene of *B. cinerea*

The described bioassay data suggest a target site modification as the mechanism responsible for resistance to benzimidazoles. In order to verify this hypothesis, a region of the β-tubulin gene from the wild-type strain (DSM-877) was analysed. A 751 bp β-tubulin cDNA fragment was amplified by using specific primers designed on the partial sequence of *B. cinerea* β-tubulin. The PCR products of the expected size were sequenced in both directions and analysed. Comparison of the deduced β-tubulin amino acid sequence with amino acid sequences of the β-tubulin gene from other fungi is shown in Fig. 2. The amino acid alignment indicates a high sequence identity of β-tubulin protein in the wild-type strain of *B. cinerea* and other ascomycetes. It is important to note the presence of glutamic acid and phenylalanine at positions 198 and 200, respectively, which characterise the benzimidazole-sensitive fungal species. Codon changes in the β-tubulin gene of field benzimidazole-resistant isolates of plant pathogenic fungi are restricted to the above codons in most fungal species (Davidse and Ishii 1995).

Identification of mutations within the β-tubulin gene of *B. cinerea*

The β-tubulin fragment from codon 109 to 418, a region known to encompass mutations conferring target-site modification responsible for benzimidazole resistance, was amplified from the resistant strains and compared to the wild-type strain. Two amino acid substitutions occurred at codon 198 (numbering according to *M. graminicola* sequence, AJ310917) in the highly benzimidazole-resistant strains compared to the wild-type strain (Table 3). The first one, a

Table 2 Fungicide sensitivity of wild-type and representative benzimidazole-resistant isolates of *Botrytis cinerea*

Fungicide	Wild-type EC_{50}[b] (μg ml^{-1}) (mean ± SD[c])	Rf[a] based on EC50[b] (mean ± SD[c])						
		BrDs-strains				BrDr-strains		
		Ben-11	Ben-26	Ben-F1	Ben-F2	Ben-4	Ben-7	Ben-12
Carbendazim	0.15±0.03	235 (±5)	240 (±3)	228 (±6)	254 (±4)	27 (±3)	23 (±2)	26 (±4)
Benomyl	0.25±0.02	220 (±4)	195 (±8)	210 (±4)	225 (±6)	19 (±2)	25 (±5)	16 (±3)
Diethofencarb	>75	<0.05	<0.01	<0.03	<0.01	1.4 (±0.18)	1.2 (±0.23)	1.0 (±0.34)
Fludioxonil	0.005±0.0007	1.1 (±0.14)	1.0 (±0.25)	1.1 (±0.12)	1.0 (±0.25)	1.3 (±0.28)	1.0 (±0.12)	1.6 (±0.12)
Iprodione	0.25 ± 0.04	1.0 (±0.22)	nt[d]	nt	1.2 (±0.54)	1.8 (±0.17)	nt	1.4 (±0.06)
Fenhexamid	0.19 ± 0.08	1.3 (±0.10)	1.0 (±0.23)	1.0 (±0.45)	1.1 (±0.11)	nt	1.7 (±0.56)	1.2 (±0.37)
Cyprodinil	0.05 ± 0.002	1.2 (±0.13)	1.2 (±0.10)	1.6 (±0.27)	1.7 (±0.37)	1.5 (±0.13)	nt	1.8 (±0.28)
Boscalid	12.5 ± 2.67	1.0 (±0.25)	1.1 (±0.12)	1.4 (±0.36)	nt	nt	1.8 (±0.22)	1.5 (±0.13)
Pyraclostrobin	0.025 ± 0.002	1.1 (±0.14)	1.3 (±0.36)	1.0 (±0.22)	nt	nt	1.6 (±0.32)	2.1 (±0.27)
Azoxystrobin	0.05 ± 0.01	1.0 (±0.13)	1.1 (±0.32)	nt	1.0 (±0.18)	nt	nt	nt
Fluazinam	0.025 ± 0.006	1.3 (±0.21)	1.5 (±0.18)	1.7 (±0.33)	nt	1.2 (±0.24)	1.0 (±0.02)	nt

[a] Resistance factor

[b] Effective concentration causing 50% reduction in mycelial growth rate after 4 days incubation at 22°C (n=3)

[c] Pooled standard deviation of the means (n = 3). Correlation coefficient ranged from 0.86 to 0.98

[d] nt: not tested

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B. cinerea(wt)      ------------------------------------------------------------
N. crassa           MREIVHLQTGQCGNQIGAAFWQTISGEHGLDASGVYNGTSELQLERMNVYFNEASGNKYV
G. fujikuroi        MREIVHLQTGQCGNQIGAAFWQTISGEHGLDSNGVYNGTSELQLERMSVNFNERSGNKYV
A. nidulans         MREIVHLQTGQCGNQIGAAFWQTISGEHGLDGSGVYNGTSDLQLERMNVYFNEASGNKYV
M. graminicola      MREIVHLQTGQCGNQIGAAFWQTISGEHGLDGSGVYNGISDLQLERMNVYFNEASGNKYV

B. cinerea(wt)      ------------------------------------------------TEGAELVDQVLDVV
N. crassa           PRAVLVDLEPGTMDAVRAGPFGQLFRPDNFVFGQSGAGNNWAKGHYTEGAELVDQVLDVV
G. fujikuroi        PRAVLVDLEPGTMDAVRAGPFGQLFRPDNFVFGQSGAGNNWAKGHYTEGAELVDQVLDVV
A. nidulans         PRAVLVDLEPGTMDCVRAGPFGELFRPDNFVFGQSGAGNNWAKGHYTEGAELVDNVVDVV
M. graminicola      PRAVLVDLEPGTMDAVRAGPFGQLFRPDNFVFGQSGAGNNWAKGHYTEGAELVDQVLDVV
                                                             ******** * ***

B. cinerea(wt)      RREAEGCDCLQGFQITHSLGGGTGAGMGTLLISKIREEFQDRMMATFSVVPSPKVSDTVV
N. crassa           RREAEGCDCLQGFQITHSLGGGTGAGMGTLLISKIREEFPDRMMATYSVVPSPKVSDTVV
G. fujikuroi        RREAEGCDCLQGFQITHSLGGGTGAGMGTLLISKIREEFPDRMMATFSVVPSPKVSDTVV
A. nidulans         RREAEGCDCLQGFQITHSLGGGTGAGMGTLLISKIREEFPDRMMATFSVVPSPKVSDTVV
M. graminicola      RREAEGCDCLQGFQITHSLGGGTGAGMGTLLISKIREEFPDRMMATFSVMPSPKVSDTVV
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B. cinerea(wt)      EPYNATLSVHQLVENSDETFCIDNEALYDICMRTLKLSNPSYGDLNHLVSAVMSGVTTCL
N. crassa           EPYNATLSVHQLVENSDETFCIDNEALYDICMRTLKLSNPSYGDLNHLVSAVMSGVTVSL
G. fujikuroi        EPYNATLSVHQLVENSDETFCIDNEALYDICMRTLKLSNPSYGDLNYLVSAVMSGVTTCL
A. nidulans         EPYNATLSVHQLVEHSDETFCIDNEALYDICMRTLKLSNPSYGDLNHLVSAVMSGVTTCL
M. graminicola      EPYNATLSVHQLVENSDATFCIDNEALYDICMRTLKFNNPSYGDLNHLVSAVMSGVTTCL
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B. cinerea(wt)      RFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGAHSFRAVTVPELTQQMYDPKIMM
N. crassa           RFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGAHHFRAVSVPELTQQMFDPKNMM
G. fujikuroi        RFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGAHSFRAVSVPELTQQMFDPKNMM
A. nidulans         RFPGQLNSDLRKWAVNMVPFPRLHFFMVGFAPLTSRGAYSFRAVSVPELTQQMFDPKNMM
M. graminicola      RFPGQLNNDLRKLAVNMVPFPRLHFFMVGFAPLTSRGAHSFRAVTVPELTQQIFDPKNMM
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B. cinerea(wt)      AASDFRNGRYLTCSAIFRGKVSMKEVEDQMRNVQNKNSSYFVEWIPNNVQTALCSIPPRG
N. crassa           AASDFRNGRYLTCSAIFRGKVSMKEVEDQMRNVQNKNSSYFVEWIPNNVQTALCSIPPRG
G. fujikuroi        AASDFRNGRYLTCSAIFRGRVAMKEVEDQMRNVQNKNSSYFVEWIPNNIQTALCAIPPRG
A. nidulans         AASDFRNGRYLTCSAIFRGKVSMKEVEDQMRNIQSKNQSYFVEWIPNNIQSALCSIPPRG
M. graminicola      AASDFRNGRYLTCSAIYRGKVSMKEVEDQIRNVQNKNTAYFVEWIPNNVQTALCSIPPRG
                    ***************** ** * ******* ** * **  ********* * *** *****

B. cinerea(wt)      LKMSSTFVGNWTSIQELFKRVGDQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDL--
N. crassa           LKMSSTFVGNSTAIQELFKRIGEQFTAMFKRKAFLHWYTGEGMDEMEFTEAESNMNDLVS
G. fujikuroi        LTMSSTFIGNSTSIQELFKRVGEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS
A. nidulans         LKMSSTFIGNSTSIQELFKRVGDQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS
M. graminicola      LKMSSTFVGNSTSIQELFKRVGDQFSAMFRRKAFLHWYTGEGMDKMEFTEAESNMNDLVS
                    * ***** ** * ******* * ** *** ************** *************

B. cinerea(wt)      ---------------------------
N. crassa           EYQQYQDAGVDEEEEEYEEEAPLEGEE
G. fujikuroi        EYQQYQDAGIDEEEEEYEEELPEGEE-
A. nidulans         EYQQYQDASISEGEEEYAEEEIMEGEE
M. graminicola      EYQQYQEASVSDAEEEYDEEAPLEGEE
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Fig. 2 Amino acid alignment of putative β-tubulin protein from wt strain (DSM877) of *B. cinerea* with other fungal proteins. Alignment was performed using Clustal W. Gaps introduced to maximise sequence identity are shown by a horizontal dash. Identical amino acids are indicated by asterisks. The two amino acid positions (198 & 200) responsible for benzimidazole resistance are shown in bold. Accession numbers: *Neurospora crassa M13630; Gibberella fujikuroi U27303; Botrytis cinerea X73133; Mycosphaerella graminicola CAC48192; Aspergillus nidulans AAA33329*

GAG-to-GCG resistance mutation, resulted in the replacement of a glutamic acid residue with an alanine in the benzimidazole-resistant Ben-26, Ben-F1 and Ben-F2 strains. The second mutation, a glutamic acid (GAG)-to glycine (GGG) replacement at the same amino acid position, was identified in the Ben-11 strain. A mutation at amino acid residue 200 (F200Y), which is also implicated in benzimidazole resistance in other fungal species, was not observed in any of these isolates. Molecular analysis of the other strains characterised by a low level of resistance (Ben-4, Ben-7, Ben-27 and Ben-12) did not reveal any mutations at this β-tubulin gene fragment (codons 109–418) (data not shown).

Diagnostic PCR-RFLP assay for detection of E198A and E198G resistant mutations

Luck and Gillings (1995) developed a diagnostic polymerase chain reaction assay to distinguish between resistant (A198) and sensitive (E198) to benzimidazole fungicides β-tubulin alleles in *B. cinerea* strains. We adapted this technique in order to detect both the E198A and the novel E198G

Table 3 Mutations found in β-tubulin gene in different strains of *Botrytis cinerea*

Strain	Description	Sequence at position 198		Sequence at position 200	
DSM 877 (WT)	Benzimidazole-sensitive	GAG	Glutamate	TTC	Phenylalanine
Ben-11	Benzimidazole-resistant	GGG	Glycine	TTC	Phenylalanine
Ben-26	Benzimidazole-resistant	GCG	Alanine	TTC	Phenylalanine
Ben-F1[a]	Field-isolate	GCG	Alanine	TTC	Phenylalanine
Ben-F2[b]	Field-isolate	GCG	Alanine	TTC	Phenylalanine
Ben(HR)NPC(S)[c]	Benzimidazole-resistant	GCG	Alanine	TTC	Phenylalanine
Ben(HR)NPC(R)[c]	Benzimidazole-resistant	AAG	Lysine	TTC	Phenylalanine
Ben(MR)NPC(R)[c]	Benzimidazole-resistant	GAG	Glutamate	TAC	Tyrosine

[a] Isolated from cucumber
[b] Isolated from strawberry
[c] Yarden & Katan (1993)

resistance mutation identified in the present study. The assay utilises a *Bsa*I restriction site found at the 196–199 amino acids in the sensitive isolate but destroyed by both E198A and E198G mutations in the laboratory and field resistant strains (Fig. 3). The digestion of PCR product from the wild-type strain, with the restriction enzyme specific for the *Bsa*I site, resulted in two bands of 98 and 371 bp. In the resistant strains the PCR product was not digested resulting in one band of 469 bp (Fig. 4).

Discussion

Resistance to benzimidazole fungicides has been detected in many fungal species in laboratory and field studies (Davidse and Ishii 1995). It is well accepted that the main mechanism of resistance in fungal pathogens to fungicides results from a modification at the target-site of fungicidal action (Brent and Hollomon 1998). The genetics of benzimidazole resistance in *Aspergillus nidulans* (van Tuyl 1977),

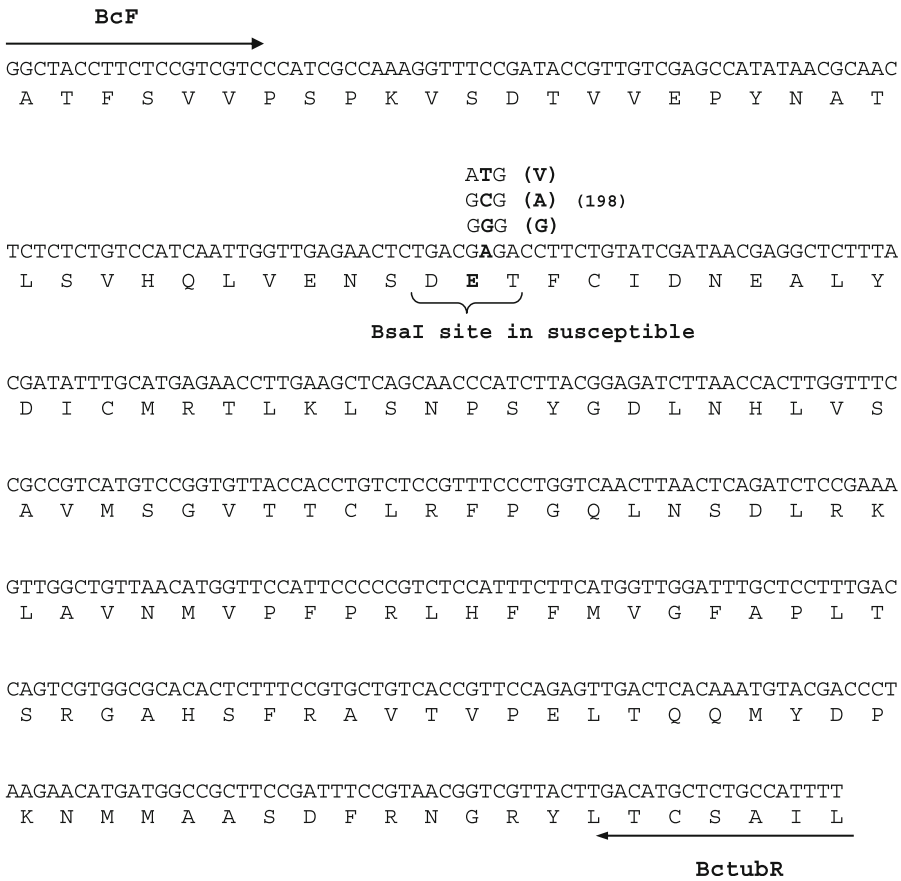

Fig. 3 Diagrammatic representation of the primer locations and mutation-associated restriction site variations in *B. cinerea* β-tubulin amplification products

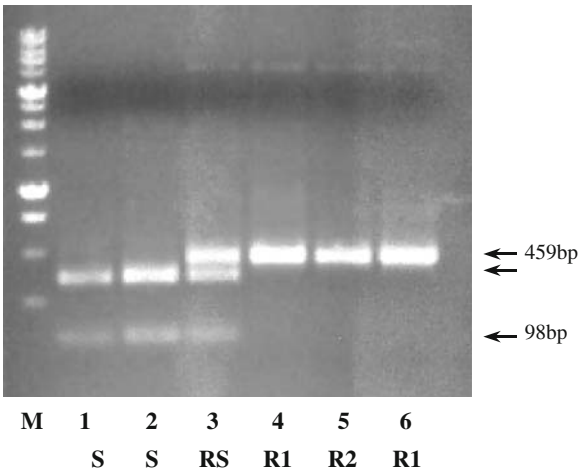

Fig. 4 PCR-RFLP detection of E198A and E198G β-tubulin-resistant mutations in *B. cinerea*. PCR products were obtained using primer set bcF and bctubR, digested with the restriction enzyme *Bsa*I, and separated on a 2.5% agarose gel containing ethidium bromide. M indicates the marker lane (1Kb). Lane 1: susceptible wild-type B (E198); Lane 2: susceptible wild-type (E198); Lane 3: 1:1 DNA mixture of S (E198) and R (G198) isolates; Lane 4: benomyl-resistant Bc-26 strain (A198), Lane 5: benomyl-resistant Bc-11 strain (G198); Lane 6: field-isolate fr

Ustilago maydis (Ziogas and Girgis 1993) and other fungal species (Ishii 1992) revealed that in most cases it was single-gene based. Early binding studies with wild-type and resistant mutants of benzimidazole-sensitive species such as *B. cinerea*, *Gibberella fujikuroi*, *Fusarium oxysporum* f.sp. *lycopersici*, *Penicillium* spp., *Rhynchosporium secalis* and *Venturia nashicola* showed that binding of carbendazim in cell-free extracts of resistant strains was low compared with that in extracts of sensitive strains (Davidse and Ishii 1995). The role of β-tubulin mutations in fungal sensitivity to both benzimidazole and phenylcarbamate fungicides has also been well established by binding assays (Hollomon et al. 1998). Molecular studies with laboratory and field isolates have shown that resistance to these fungicides resulted from a target-site modification through point mutations in the β-tubulin gene, that alter amino acid sequences at the benzimidazole-binding site (Davidse and Ishii 1995).

In the present study, mutants of *B. cinerea* resistant to benzimidazoles, with or without sensitivity to the phenylcarbamate diethofencarb, were isolated after chemical mutagenesis and selection on medium containing carbendazim or a mixture of carbendazim and diethofencarb. Studies of the sensitivity of mutant strains to other fungicides showed that the mutation(s) for resistance to carbendazim also reduced the sensitivity of mutant strains to the benzimidazole benomyl but not to the phenypyrrole fludioxonil, the dicarboximide iprodione, the hydroxyanilide fenhexamid, the anilinopyrimidine cyprodinil, the phenylpyridinamine fluazinam, the carboxamide boscalid, and to the Qo-inhibiting fungicides azoxystrobin and pyraclostrobin, which affect other cellular pathways. Studies of fitness-determining characteristics in wild-type and representative laboratory and field benzimidazole-resistant isolates of *B. cinerea*, showed that the mutation(s) leading to high carbendazim resistance did not carry fitness penalties (Table 1). The non-effect or low effect of benzimidazole-resistant mutations on ecological fitness gives a satisfactory explanation for the persistence of resistance phenomenon in the field long after the withdrawal of benzimidazole fungicides (Georgopoulos and Skylakakis 1986).

Molecular analysis of the β-tubulin gene in low-level resistant mutants (Ben-4, Ben-12, Ben-27) did not reveal any amino acid substitutions. Apparently, mechanisms other than target-site modification such as overproduction of the fungicide target, a metabolic breakdown of the fungicide, an active efflux or reduced uptake of the fungicide, are responsible for a low-resistant phenotype (Brent and Hollomon 1998).

On the contrary, analysis of the β-tubulin gene in the highly benzimidazole-resistant strain (Ben-11) revealed a novel resistance mutation from glutamic acid to glycine at the codon 198 (E198G). The E198G resistance mutation has previously been reported in *Neurospora crassa* (Fujimura et al. 1992), *Venturia inaequalis* (Koenraadt et al. 1992), *R. secalis* (Koenraadt et al. 1992) and the cereal eyespot fungal pathogens *Tapesia yallundae* and *T. acuformis* (Albertini et al. 1999). The glutamic acid to alanine mutation at the same codon (E198A) was also found in the resistant strain Ben-26 and two benzimidazole-resistant field isolates (Ben-F1, Ben-F2). The role of this mutation has previously been recognised in benzimidazole resistance in *B. cinerea* (Yarden and Katan 1993; Leroux et al. 2002), *N. crassa* (Orbach et al. 1986; Jung et al. 1992), *Penicillium expansum* (Fujimura et al. 1992), *V. inaequalis* and *V. pirina* (Fujimura et al. 1992), *P. aurantiogriseum* (Koenraadt et al. 1992) and *Monilinia fructicola* (Ma et al. 2003).

Molecular technology gives us the potential for developing molecular diagnostics for fast and accurate monitoring and management of fungicide resistance. Towards this goal, an adaptation of the diagnostic PCR-RFLP method by Luck and Gillings (1995) was developed to detect both the novel (E198G) resistance mutation and the known (E198A) in benzimidazole-resistant strains of *B. cinerea*. Luck and Gillings (1995) utilised the creation of a *Tha*I (now called *Bst*U1) restriction site at codon 198 in benomyl-resistant strains where the amplification products were successfully digested, while PCR products from benzimidazole-sensitive strains remained undigested. However, this assay is unable to detect the novel E198G mutation described in our study. Therefore, an adaptation of the PCR-RFLP approach was developed by utilising the *Bsa*I restriction site present in the wild-type strain but destroyed by both E198A and E198G resistance mutations. This molecular diagnostic assay might be more valuable for the monitoring of *B. cinerea* benzimidazole resistance in the field, especially if the E198G mutation is present in field-resistant isolates.

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References

Albertini, C., Gredt, M., & Leroux, P. (1999). Mutations of the β tubulin gene associated with different phenotypes of benzimidazole resistance in the cereal eyespot fungi *Tapeia yallundae* and *Tapesia acuformis*. *Pesticide Biochemistry and Physiology, 64*, 17–31. doi:10.1006/pest.1999.2406.

Banno, S., Fukumori, F., Ichiishi, A., Okada, K., Uekusa, H., Kimura, M., et al. (2008). Genotyping of Benzimidazole-Resistant and Dicarboximide-Resistant Mutations in *Botrytis cinerea* Using Real-Time Polymerase Chain Reaction Assays. *Phytopathology, 98*, 397–404. doi:10.1094/PHYTO-98-4-0397.

Brent, K. J., & Hollomon, D. W. (1998). *Fungicide Resistance: The assessment of risk. FRAC Monograph No 2*, pp. 1–48. Brussels: Global Crop Protection Federation.

Davidse, L., & Ishii, T. (1995). Biochemical and molecular aspects of benzimidazoles, N-phenylcarbamates and N-phenylformamidoxines and the mechanisms of resistance to these compounds in fungi. In H. Lyr (Ed.), *Modern selective fungicides*, pp. 305–322. Jena, Germany: Gustav Fisher.

Faretra, F., Pollastro, U. S., & Ditonno, A. P. (1989). New natural variants of *Botryotinia fackeliana* (*Botrytis cinerea*) coupling benzimidazole-resistance to insensitivity toward the N-phenylcarbamate diethofencarb. *Phytopathologia Mediterranea, 28*, 97–104.

Fujimura, M., Kmakura, T., Inoue, S., & Yamaguchi, I. (1992). Sensitivity of *Neurospora crassa* to benzimidazoles and N-phenylcarbamates: effect of amino acid substitutions at position 198 in β tubulin. *Pesticide Biochemistry and Physiology, 44*, 165–167. doi:10.1016/0048-3575(92)90087-G.

Georgopoulos, S. G., & Skylakakis, G. (1986). Genetic variability in the fungi and the problem of fungicide resistance. *Crop Protection (Guildford, Surrey), 5*, 299–305. doi:10.1016/0261-2194(86)90107-9.

Gullino, M. L., & Garibaldi, A. (1986). Resistance to fungicides in *Botrytis cinerea*. Present situation. *Notiziario Malattie Delle Plante, 107*, 63–71.

Hollomon, D. W., Butters, J. A., Barker, H., & Hall, L. (1998). Fungal *β*-tubulin, expressed as fusion protein, binds benzimidazole and phenylcarbamate fungicides. *Antimicrobial Agents and Chemotherapy, 9*, 2171–2173.

Ishii, H. (1992). Target sites of tubulin-binding fungicides. In W. Koller (Ed.), *Target Sites of Fungicide Action*, pp. 43–52. Boca Raton: CBC.

Jung, M. K., Wilder, I. B., & Oakley, B. R. (1992). Amino acid alterations in the benA (Beta-tubulin) gene of *Aspergillus nidulans* that confer benomyl resistance. *Cell Motility and the Cytoskeleton, 22*, 170–174. doi:10.1002/cm.970220304.

Katan, T., Elad, Y., & Yunis, H. (1989). Resistance to diethofencarb (NPC) in benomyl-resistant field isolates of *Botrytis cinerea*. *Plant Pathology, 38*, 86–92. doi:10.1111/j.1365-3059.1989.tb01431.x.

Kato, T. K., Suzuki, J., Takahashi, K., & Kamoshita, K. (1984). Negatively correlated cross-resistance between benzimidazole fungicides and methyl N-(3, 5-dichlorephenyl)-carbamate. *Journal of Pest Science, 9*, 489–495.

Koenraadt, H., Somerville, S. C., & Jones, A. L. (1992). Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. *Phytopathology, 82*, 1348–1354. doi:10.1094/Phyto-82-1348.

Laskaris, D., Pappas, A. C., & Kyriakopoulos, C. K. (1996). Occurrence of *Botrytis cinerea* Pers. ex Fr. strains with coupling resistance to benzimidazoles and phenylcarbamates in protected crops in Greece. *Phytopathologia Mediterranea, 35*, 223.

Leroux, P., & Gredt, M. (1989). Negative cross-resistance of benzimidazole resistant strains of *Botrytis cinerea, Fusarium nivale* and *Pseudocercosporella herpotrichoides* to various pesticides. *Netherlands Journal of Plant Pathology, 95*, 121–127. doi:10.1007/BF01974291.

Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., et al. (2002). Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. *Pest Management Science, 58*, 876–888. doi:10.1002/ps.566.

Lorenz, G. (1988). Dicarboximide fungicides: history of resistance development and monitoring methods. In C. J. Delp (Ed.), *Fungicide Resistance in North America*, pp. 44–51. St Paul, MN, USA: American Phytopathological Society.

Luck, J. E., & Gillings, M. R. (1995). Rapid identification of benomyl resistant strains of *Botrytis cinerea* using the

polymerase chain reaction. *Mycological Research, 99*, 1483–1488.

Ma, Z., Yoshimura, M., & Michailides, T. J. (2003). Identification and characterization of benzimidazole resistance in *Monilinia fructicola* from Stone Fruit Orchards in California. *Applied and Environmental Microbiology, 69*(12), 7145–7152. doi:10.1128/AEM.69.12.7145-7152.2003.

Malathrakis, N. E. (1979). Studies on gray mold (*Botrytis cinerea*) of vegetables grown under plastics. *Phytopathologia Mediterranea, 19*, 70.

Moorman, G. W., & Lease, R. J. (1992). Benzimidazole and dicarboximide-resistant *Botrytis cinerea* from Pennsylvania greenhouses. *Plant Disease, 76*, 477–480.

Orbach, M. J., Porro, E. B., & Yanofsky, C. (1986). Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. *Molecular and Cellular Biology, 6*, 2452–2461.

Panayotakou, N., & Malathrakis, N. E. (1983). Resistance of *Botrytis cinerea* to dicarboximide fungicides in protected crops. *Annals of Applied Biology, 102*, 293–299. doi:10.1111/j.1744-7348.1983.tb02696.x.

Raposo, R., Delcan, J., & Melgarejo, P. (1994). Multiple fungicide resistance in *Botrytis cinerea* from commercial greenhouses in southern Spain. In: *Proceedings of Brighton Crop Protection Conference, Pests and Diseases* (pp. 493–498). UK: British Crop Protection Council, Surrey.

Smith, C. M. (1988). History of benzimidazole use and resistance. In C. J. Delp (Ed.), *Fungicide Resistance in North America*, pp. 23–24. St Paul, MN, USA: American Phytopathological Society.

Stehmann, C., & de Waard, M. A. (1996). Sensitivity of populations of *Botrytis cinerea* to triazoles, benomyl, and vinclozolin. *European Journal of Plant Pathology, 102*, 171–180. doi:10.1007/BF01877104.

van Tuyl, J. M. (1977). Genetics of fungal resistance to systemic fungicides. *Mededelingen Landbouwhogeschool Wageningen, 77*, 1–136.

Yarden, O., & Katan, T. (1993). Mutations leading to substitutions at amino acid 198 and 200 of beta–tubulin that correlate with benomyl-resistant phenotypes of field strains of *Botrytis cinerea*. *Phytopathology, 83*, 1478–1483. doi:10.1094/Phyto-83-1478.

Ziogas, B. N., & Girgis, S. M. (1993). Cross-resistance relationships between benzimidazole fungicides and diethofencarb in *Botrytis cinerea* and their genetical basis in *Ustilago maydis*. *Pesticide Science, 39*, 199–205. doi:10.1002/ps.2780390306.