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Detection and characterization of benzimidazole resistance of *Botrytis cinerea* in greenhouse vegetables

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Abstract From 2003 to 2006, a total of 426 single-conidial isolates of *B. cinerea* collected from greenhouse vegetables in China were characterized for resistance to benzimidazole fungicides and diethofencarb according to inhibition of mycelial growth. Rapid development of double-resistance to benzimidazoles and diethofencarb was observed. Three types of benzimidazole-resistant isolates, Ben R1, Ben R2 and Ben R3 were detected. A new phenotype, Ben R3, which showed low level of resistance to benzimidazole fungicides and resistance to diethofencarb, was detected with frequencies of 6.8%, 10.0%, 13.2% and 12.4% from 2003 to 2006, respectively. Further studies indicated that Ben R3 was caused by a point mutation from GAG in sensitive(S) isolates to GTG at codon 198 in the β-tubulin gene, predicted to cause a change from glutamic acid to valine. Ben R3 isolates had comparable growth, sporulation and pathogenicity ability as isolates of other phenotypes but were more sensitive at lower temperatures.

Keywords *Botrytis cinerea* · Vegetable grey mould · Benzimidazole resistance · β-tubulin gene · Double resistance

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Introduction

Botrytis cinerea, the causal agent of grey mould disease, is a ubiquitous plant pathogenic fungus worldwide (Rosslenbroich and Stuebler 2000). Grey mould is one of the most destructive diseases in greenhouse vegetables since it severely reduces the yield and quality in crops such as eggplant, tomato, cucumber and pepper (Prins et al. 2000). In China, control of grey mould is based on the use of fungicides applied in 7-d intervals from November to May. Negative cross-resistance between the benzimidazole and *N*-phenylcarbamate (NPC) fungicides has been observed in *B. cinerea* (Elad et al. 1988; Leroux 1992; Leroux et al. 2002). In China, the mixture (carbendazim and diethofencarb) has been extensively applied since the late 1990s.

Resistance to benzimidazole fungicides has been reported in China since 1994 (Zhang et al. 2007; Zhou

et al. 1994). Resistance of *B. cinerea* to benzimidazoles has been detected in Europe (Beever et al. 1989; Dianez et al. 2002; Leroux et al. 2002; Pappas 1997), Asia (Yarden and Katan 1993) and Canada (Noethover and Matteoni 1986). However, only two types of benzimidazole-resistant isolates, Ben R1and Ben R2, were widely detected in previous field studies. Ben R1 isolates, caused by a point mutation from GAG in sensitive(S) isolates to GCG at codon 198 in the β-tubulin gene, are highly resistant to benzimidazoles and simultaneously more sensitive to the phenyl-carbamate diethofencarb than the wild type isolates. Ben R2 isolates, caused by a point mutation from TTC in sensitive(S) isolates to TAC at codon 200 in the β-tubulin gene, are moderately resistant to benzimidazoles and insensitive to diethofencarb, just like the wild type benzimidazole-sensitive isolates. Ben R2 isolates were detected after the introduction of the mixture of carbendazim and diethofencarb (Leroux et al. 2002; Yarden and Katan 1993; Zhang et al. 2003).

Resistance to benzimidazole fungicides has been reported in many fungal species. In most cases, resistance is associated with point mutations in β-tubulin gene which result in altered amino acid sequences at the benzimidazole binding site (Davidson et al. 2006; Koenraadt et al. 1992; Ma et al. 2003; Maymon et al. 2006). Most field resistant isolates of plant pathogenic fungi show codon changes that seem to be restricted to positions 50 (McKay et al. 1998), 198, 200 (Albertini et al. 1999; Koenraadt et al. 1992), and 240 (Albertini et al. 1999). Only a few exceptions to these point mutations have been reported in *Venturia inaequalis*, *Penicillium expansum*, *Penicillium aurantiogriseum* (Koenraadt et al. 1992), *Gibberella pulicaris* (Kawchuk et al. 2002), and *Gibberella zeae* (Chen et al. 2005), but the exact molecular mechanisms for their resistances have not been clarified.

Mutations in the β-tubulin gene may have pleiotropic effects on fungal growth at high or low temperatures (Davidse 1986). Several benomyl-resistant mutants of model fungi exhibit temperature-sensitive phenotypes. For plant pathogenic fungi, only low resistance isolates of *Monilinia fructicola* which were sensitive at low temperatures and low resistance isolates of *M. laxa* which were sensitive at high temperatures have been reported (Ma et al. 2003, 2005). Ma speculated that temperature sensitivity of different benzimidazole resistant isolates could be used to manage resistance but no methodology was reported.

The current study was conducted to (i) monitor the evolution of resistance to benzimidazole fungicides and diethofencarb of *B. cinerea* on greenhouse vegetables in China, (ii) determine the temperature sensitivity in benzimidazole-resistant and -sensitive isolates of *B. cinerea*, and (iii) investigate molecular mechanism of benzimidazole resistance of *B. cinerea*.

Materials and methods

Fungicides

Technical grade diethofencarb and benzimidazole fungicides, including carbendazim and thiophanate-methyl, were dissolved in acetone except that carbendazim was dissolved in 0.1 mol l^{-1} hydrochloric acid (HCl) to prepare the stock solutions (Zhang et al. 2007).

Single-conidial isolates of *B. cinerea*

From 2003 to 2006, a total of 426 single-conidial isolates were collected as described previously (Zhang et al. 2007) from 32 commercial greenhouses of vegetable crops (strawberry, tomato, eggplant and pepper) located in Zhejiang and Jiangsu provinces, which are the major vegetable production regions in China (Table 1). Five to ten isolates were collected from each greenhouse. In the sampled greenhouses, the most frequently used fungicides were the mixture of carbendazim and diethofencarb, and dicarboximide fungicides.

Quantitative assessments of the fungicide sensitivity of *B. cinerea*

Inhibition of mycelia growth was assessed through measuring the radial growth on solid PDA plates amended with 0, 0.0125, 0.05, 0.2, 1, 5, 10, 25, 50, 100, 200 and 500 mg a.i. l^{-1} of medium (Yarden and Katan 1993; Leroux et al. 2002) according to the method described previously (Zhang et al. 2007). For each isolate, the average colony diameters measured in two perpendicular directions was used to calculate the EC_{50} (the fungicide concentration that results in 50% radial growth inhibition) by linear regression of probit % inhibition of radial growth as a function of the $\log_{10}$

Table 1 Number of isolates of *Botrytis cinerea* from each year, region and host crop tested for fungicide resistance

Host	Zhejiang Province				Jiangsu Province			
	2003	2004	2005	2006	2003	2004	2005	2006
Strawberry	6	16	10	8	21	13	23	10
Tomato	8	21	12	12	15	17	15	12
Eggplant	10	26	21	15	16	8	19	16
Pepper	6	8	13	8	6	11	16	8
Total	30	71	56	43	58	49	73	46

fungicide concentration. The experiment was performed twice.

Test of temperature sensitivity of *B. cinerea*

To determine the temperature sensitivity of *B. cinerea*, 10 isolates were chosen at random for each phenotype of benzimidazole-sensitivity. Each of the 40 isolates was tested for its ability to grow at various temperatures on PDA amended with 0,1, 5, 10, 50, 100, and 200 mg a.i. l^{-1} of medium as the method described by Ma et al. (2003) Three replicates of each fungicide concentration were used. After respective incubation at 10, 16, 22, 28, and 34°C for 10 d in the dark, radial growth was measured for each plate. The experiment was performed twice.

Characterization of Ben R3 isolates

Five isolates for each phenotype of benzimidazole sensitivity were chosen at random to compare some important biological characteristics; hyphal growth, sporulation, and spore germination according the method described previously (Zhang et al. 2007). Pathogenicity was determined according the method described by Elad (1992). Eggplant plants were grown in pots in greenhouses with a minimum temperature of 15°C. After about 30 d, each developed leaf was inoculated with one mycelium disc. The discs were cut from the margin of a 6-d-old colony and were placed in the centre of leaves. To create favourable conditions for infection, inoculated plants were maintained in the dark with 95% humidity at 23°C for 24 h, and then moved back into the plant growth chambers. The plants were then kept at 23°C with 85% humidity and 12 h light/dark cycle. Five days after inoculation, lesion development from mycelium discs was determined by measuring two diameters at right angles. Three plants for each strain were used and the experiment was performed twice. Mean lesion diameters were calculated to represent the development of disease.

Isolation the β-tubulin gene fragments of *B. cinerea*

Eight isolates were chosen at random for each phenotype of benzimidazole sensitivity. To extract DNA, each isolate was grown at PDA plates for 5 d in the dark. Mycelia were harvested and washed in sterile water, frozen in liquid nitrogen, and lyophilized. DNA from each isolate was extracted by using a CTAB method (Kachroo et al. 1995). According the known complete sequence of the β-tubulin gene in *B. cinerea* (GenBank accession number U27198), the PCR primer pair Bcb-F (5'-CACTGAGGGTGCTGAGCTTGT-3') + Bcb-R (5'-GAAGCGGCCATCATGTTCTTA-3') was designed to amplify the β-tubulin gene fragment containing the codon 198 and 200. All PCR reactions were performed in 50 μl volumes and contained 100 ng of template DNA, 5 μl 10 × Taq DNA polymerase reaction buffer, 2.5°C$mmol^{-1}$ dNTPs, and 1.4°CU Taq DNA polymerase (Invitrogen, Shanghai, China). The PCR program was 94°C × 5 min, (94°C × 1 min, 53°C × 1 min, 72°C × 1 min) × 35 cycles, followed by a final extension at 72°C × 10 min. PCR products were analyzed by agarose gel electrophoresis, and purified using 3 S Spin Agarose Gel DNA Purification Kit (Shenerge Biocolor Company, Shanghai, China) following the protocol of the manufacturer. All PCR products were sequenced by Scigene Company, Shanghai, China. Sequences were aligned by using the software Clustal W (http://www.ebi.ac.uk).

Data analysis

EC_{50} values were calculated by linearly regression of probit % of inhibiting radial growth as a

function of the log of inhibitor concentrations. Multiple comparison tests (least significant difference, LSD) were used to detect differences among means such as EC_{50} values. Statistical tests were performed using SPSS (Statistical Product and Service Solutions), version 11.0.

Results

Benzimidazole resistance and diethofencarb sensitivity of *B. cinerea* greenhouse populations

Three different levels of benzimidazole resistance were detected in the tested populations. Sensitive(S) isolates could not grow on 1 mg l^{-1} carbendazim or thiophanate-methyl and had EC_{50} values <0.1 mg l^{-1}. Low resistance (LR) isolates could grow on 5 mg l^{-1} but could not on 10 mg l^{-1} carbendazim or thiophanate-methyl and had EC_{50} values ranging from 0.8 to 8.2 mg l^{-1}. Moderate resistance (MR) isolates could grow on 50 mg l^{-1} but not on 100 mg l^{-1} carbendazim or thiophanate-methyl and had EC_{50} values ranging from 15.4 to 22.6 mg l^{-1}. High resistance (HR) isolates can grow on 200 mg l^{-1} carbendazim or thiophanate-methyl and had EC_{50} values >50 mg l^{-1}. From 2003 to 2006, the total resistance frequency was 62.5%, 71.7%, 77.5%, and 80.9% respectively. This suggested that severe resistance of *B. cinerea* to benzimidazoles was widespread in greenhouse vegetables in eastern China.

Two different levels of sensitivity to diethofencarb were detected. They were sensitive (S) and resistant (R). S isolates could not grow on 25 mg l^{-1} diethofencarb and had EC_{50} values ranging from 0.01 to 16.5 mg l^{-1}. R isolates could grow on 100 mg l^{-1} diethofencarb. Close association between benzimidazole resistance and diethofencarb sensitivity was found. All the diethofencarb S isolates were benzimidazole HR isolates (hereafter referred to as Ben R1 isolates). Sensitivity of the diethofencarb R isolates to benzimidazole fungicides was divided into three types, benzimidazole S, LR, and MR isolates, respectively. Hereafter, benzimidazole S- diethofencarb R isolates were referred to as Ben S isolates. Benzimidazole MR- diethofencarb R isolates were referred to as Ben R2 isolates. Benzimidazole LR-diethofencarb R isolates were referred to as Ben R3 isolates. From 2003 to 2006, the frequency of double resistance to benzimidazoles and diethofencarb was 27.3%, 38.3%, 46.5%, and 50.6% respectively.

Sensitivity of Ben S, R1, R2 and R3 isolates to low and high temperature

After incubation at various temperatures for 10 d, the Ben R1, R2, and R3 isolates showed similar high-temperature sensitivity on PDA amended with carbendazim. At 34°C, no isolate could grow on PDA amended with 1 mg l^{-1} carbendazim. At 16°C to 28°C, all isolates showed the same phenotypes of mycelial growth as those on 22°C. However, only Ben R3 isolates could not grow on PDA amended with 1 mg l^{-1} carbendazim at 10°C (Fig. 1). This suggested that Ben R3 isolates could not express their resistance at low temperatures.

Growth, sporulation and pathogenicity of Ben S, R1, R2 and R3 isolates

There were significant differences between isolates in growth, sporulation and pathogenicity ($P<0.05$). However, there were no significant differences between phenotypes in growth, sporulation and pathogenicity (Table 2).

Fragments of the β-tubulin gene of *B. cinerea*

The sequenced fragment of the β-tubulin gene of *B. cinerea* includes the positions in *B. cinerea* known to affect the sensitivity to benzimidazole fungicides. The sequenced Ben S isolates show 100% homology to

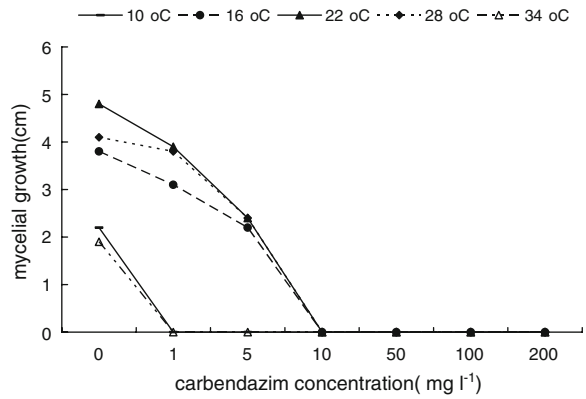

Fig. 1 Effect of temperature on mycelial growth of Ben R3 isolates of *B. cinerea* on PDA plates amended with carbendazim

Table 2 Growth rate, sporulation, germination and pathogenicity of Ben S, R1, R2, and R3 isolates of *Botrytis cinerea*

Phenotype	Colony diameter (cm)	Spores ($\times 10^6$ ml^{-1})	Germination (%)	Disease lesion diameter (cm)
Ben S	4.38a[X]	2.18 a[Y]	98.2a	1·74 a
Ben R1	4·68 a	3·20a	98.8a	3·26 a
Ben R2	5·62a	2·14 a	95.6a	3·18 a
Ben R3	5.34a	2.52 a	98.2a	2.32 a

[X] Mean of five isolates for each phenotype

[Y] Figures followed by the same letter within a column were not significantly different with LSD (least significant difference) test at P=0·05

that for *B. cinerea* in Genbank (accession number U27198). As expected with previous reports, all the Ben S, Ben R1, and Ben R2 isolates had the respective sequence GAG (glutamic acid), GCG (Alanine), and GAG (glutamic acid) at the codon 198 of β-tubulin gene and TTC (Phenylalanine), TTC (Phenylalanine), and TAC (Tyrosine) at the codon 200 of β-tubulin gene. All the Ben R3 isolates, a new phenotype, had the sequence GTG (Valine) at the codon 198 and TTC (Phenylalanine) at the codon 200 of β-tubulin gene. Apart from the differences at codon 198 and 200, all isolates had identical sequences.

Discussion

One of the objectives of this study was to monitor the development of double-resistance to benzimidazole fungicides and diethofencarb, an *N*-phenylcarbamate which has negative cross-resistance with benzimidazoles (Elad et al. 1988; Leroux 1992; Leroux et al. 2002). Frequent reports of control failure for carbendazim in China could be attributed to the dominance of the benzimidazole-resistant sub-population. Three different resistance levels, Ben R1, Ben R2, and Ben R3, were detected in *B. cinerea* isolates from greenhouse vegetables with the total resistance frequency of 62.5%, 71.7%, 77.5%, and 80.9% from 2003 to 2006 respectively. Among them, Ben R1 isolates are highly resistant to benzimidazoles and simultaneously more sensitive to diethofencarb than the wild type isolates. Ben R2 isolates are moderately resistant to benzimidazoles and insensitive to diethofencarb, just like the wild benzimidazole sensitive isolates. These two levels of resistant isolates of *B. cinerea* were extensively detected in Europe (Leroux et al. 2002) and China (Zhou et al. 1994; Zhang et al. 2006). Although three benzimidazole-resistant phenotypes had been recovered in laboratory mutants in *B. cinerea* (Ziogas and Girgis 1993), Ben R3 field isolates of *B. cinerea*, which have low resistance to benzimidazoles and are insensitive to diethofencarb, is reported for the first time.

The negative cross-resistance between the benzimidazoles and *N*-phenylcarbamate (NPC) fungicides (Elad et al. 1988) has resulted in the mixture of carbendazim and diethofencarb being extensively used since the late 1990s in China. Two different phenotypes of double-resistance to benzimidazoles and diethofencarb, Ben R2 and Ben R3, were detected in this study. From 2003 to 2006, the rapid development of double-resistance was observed with the respective frequency of 27.3%, 38.3%, 46.5%, and 50.6%. This suggests that the mixture of carbendazim and diethofencarb should not be used to control *B. cinerea* in greenhouse vegetables in China without reasonable resistance management strategies.

Several benomyl resistant mutants of model fungi exhibit temperature sensitive phenotypes (Davidse 1986). All these mutants might have decreased fitness under field conditions. However, for plant pathogenic fungi, such effects have only been recently reported for *Monilinia fructicola* and *M. laxa* (Ma et al. 2003, 2005). In *M. fructicola*, isolates with low and high resistance to benzimidazole fungicides were sensitive under low and high temperature conditions respectively. In this study, all resistant isolates, including Ben R1, Ben R2, and Ben R3 did not show resistance at high temperature (34°C). Only Ben R3 isolates were sensitive at low temperature (10°C). The difference in sensitivity of low resistance and high resistance isolates of *M. fructicola* to temperatures could be used for control stone fruit brown rot in California (Ma et al. 2003, 2005). Our study indicates

that the application of this difference in *B. cinerea* in greenhouse vegetables needs further research.

Analysis of the β-tubulin gene of *B. cinerea* has shown that Ben R1 and Ben R2 isolates had the same resistance mechanisms as previously reported (Yarden and Katan 1993; Leroux et al. 2002). In Ben R1 isolates, a point mutation from GAG in sensitive(S) isolates to GCG resulted in an alanine replacing the glutamic acid at codon 198. In Ben R2 isolates, a point mutation at codon 200 from TTC in sensitive(S) isolates to TAC resulted in a tyrosine replacing the phenylalanine. For the new phenotype, Ben R3 isolates, a point mutation at codon 198 from GAG in sensitive(S) isolates to GTG resulting in valine replacing glutamic acid, was detected in all eight sequenced Ben R3 isolates. The GAG to GTG point mutation at codon 198 of β-tubulin gene has been detected in *Penicillium* spp. (Sholberg et al. 2005) and *Venturia inaequalis* (Koenraadt et al. 1992), both resulting in high resistance to benzimidazoles.

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