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Sensitivity of *Corynespora cassiicola* to Carbendazim and Diethofencarb

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During 2005 and 2011, 159 isolates of *Corynespora cassiicola* were collected from 10 different provinces in China, and their sensitivities to carbendazim and diethofencarb were determined. Among all isolates, 8.2% were sensitive to carbendazim (M^S), 91.8% were highly resistant to carbendazim (M^{HR}) and 14.5% were highly resistant to both carbendazim and diethofencarb ($M^{HR}N^{HR}$), moderately resistant isolates to carbendazim were not found. This is the first report of *C. cassiicola* isolates that were highly resistant to both carbendazim and diethofencarb. As compared to M^S isolates, M^{HR} and $M^{HR}N^{HR}$ isolates showed similar fitness in fungal growth rate, sporulation and pathogenicity. Carbendazim resistance correlated with a single mutation in β–tubulin gene *C. cassiicola* amplified with primer pair β–tubf1-F and β–tubf1-R causing a change of glutamic to alanine at amino acid 198. Furthermore, the substitution of glutamic acid to lysine and phenylalanine to tyrosine led the resistance to both fungicides at amino acid 198 and 200 repecetively.

Key words: *Corynespora cassiicola*, Carbendazim, Diethofencarb, β–tubulin.

Corynespora cassiicola (Berk. & Curt.) Wei, a plant pathogenic fungus, has been recorded in over 70 countries and more than 280 plant species have been known to host this fungus, including fruits, vegetables, grains, perennial crops, forestry and various ornamental plants[1]. It has been known for a long time in China but outbreaks were not very serious in cucumber and other crops until several years ago. In recent years, it becomes the most important folial disease of cucumber in China[2, 3].

Control of cucumber disease largely relies on fungicides worldwide[4], so application of fungicides therefore plays an important role in this disease control. The benzimidazole fungicides carbendazim and N–phenylcarbamate fungicides diethofencarb, which are known to inhibit microtubule assembly in pathogens, have been widely used in disease control[5, 6]. Negative cross–resistance between benzimidazole fungicides and diethofencarb has been observed in *C. cassiicola*, they have been used extensively in management of this disease In Japan[7]. However, it is well known that site-specific fungicides generally possess a high risk of resistance development if resistant isolates of the pathogen are not impaired in their ability to survive and multiply in the agricultural environment, so their effectiveness decreased by occurrence of resistant strains of the pathogen[8].

Resistance to carbendazim and diethofencarb have been reported in many fungal species, the mechanism of resistance to the two fungicides is often due to the mutations in the β-tubulin gene, which result in altered amino acid sequences at the fungicide–binding site [9, 10].

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There have not been any registered fungicides for control of the disease caused by *C. cassiicola* in China and the resistance of the disease hasn't been systematic study. Because early control of the disease is essential for disease management, growers must determine quickly whether, and to what extent, fungicide resistance is present in the fungal population for effective fungicide use in management programmes.

The objective of this study was to determine the sensitivity of *C. cassiicola* isolates to carbendazim and diethofencarb and to investigate molecular mechanism of carbendazim and diethofencarb resistance of *C. cassiicola*.

MATERIALS AND METHODS

Fungicides

Technical–grade carbendazim (98.0% active ingredient; YangNong Chemicals, Jiangsu, China) and diethofencarb (95% active ingredient; Sumitomo Chemical, Shanghai, China) were dissolved in acetone to provide stock solutions containing 10,000 µg/ml. The fungicides were stored at 4°C in the dark to maintain and reserve fungicide activity.

Fungal isolates

For determining sensitivity to carbendazim and diethofencarb, a total of 159 isolates were collected from cucumber, tomato, eggplant, balsam pear, cowpea, kidney bean rubber,pepper and jasmine from 10 different regions in China during 2005–2011 (Table 1). All the isolates were obtained by single spore isolation and matained on PDA slants at 5°C until use.

Sensitivity of *C.cassiicola* to carbendazim and diethofencarb

The sensitivity test was performed by transferring a 4mm diameter disc of a colony grown on a PSA plate medium amended with 0, 0.1, 1, 10, 100 µg/ml carbendazim or diethofencarb. After incubation at 25°C in the dark for 4 days, the diameter of the mycelial colony was measured[7]. For each isolate, three replicates per concentration were used and the experiments were performed twice. For the rapid assessment of levels of resistance to carbendazim and diethofencarb,isolates were categorized depending on their sensitivity to two fungicides as described previously[8]: as sensitive (MIC values lower than 10µg/ml carbendazim or diethofencarb), moderately resistant (MIC values between 10–100µg/ml carbendazim), highly resistant(MIC values higherthan 100 µg/ml carbendazim or diethofencarb).

Determination of fitness of resistant isolates

Mycelial growth rate, sporulation and phytogenicity as the fitness patameters of carbendazim–resistant and carbendazim and diethofencarb double–resistant isolates. To determine fungal growth rate, mycelial plugs of each isolate were inoculated in the centre of PSA plates without fungicides. After incubation at 28°C for 8 days in dark, colony diameter of each isolate was measured. Five plates for each isolates were used, and the experiment was repeated two times. To determine conidial production, the fungus was cultured on PSA media for 12 days at 28°C and suspension was made by addition of 5 ml of sterilized water in each plate. Five plates for each isolates were used, and the experiment was repeated two times.

Pathogenicity of *C. cassiicola* isolates was determined by examing the lesions caused by each strain on tomato leaves. Tomato plants were grown in pots in greenhouses at 25°C. After about 40 days, the leafs were inoculated with spore suspensions with the concentration of 1×10^5 spores/ml. To create favourable conditions for infection, inoculated plants were maintained with high humidity (almost 100%) at 28°C for 2 days in the dark, and then transferred to a phytotron at 28°C under natural light conditions. Three days after inoculation, the disease severity was rated on the following indices: 0 (no symptom), 1 (infection with less than 5% of leaf area), 2 (infection with 6%–25%), 3 (infection with 26–50), 4 (infection with more than 51%).60 plants for each strain were used and the experiment was performed twice.

DNA extraction and PCR amplification of β-tubulin from *C.cassiicola*

DNA was extracted with sodium dodecylsulfate (SDS) detergentlysis buffer, followed by a phenol/chloroform extraction and precipitation in ethanol with sodium acetate[11].

Thirteen isolates were used for DNA analysis of the β-tubulin gene. Partial β-tubulin gene fragment was amplified by β-–tubf1–F (5'–CAGCTCGAGCGTATG AACGTCT–3') and β-tubf1–R (5'–TGTACCAATGCAAGAAAGCCTT–

3')[12]. All amplified PCR productions were purified by electrophoresis and cloned into the pMD 20–T Vector (TaKaRa Biotechnology Co., Ltd., Dalian, China) and sequenced by Shanghai Sangon Biological Engineering Technology And Service Co., Ltd in both directions. Sequence data were analyzed using DNAman software.

Statistical analysis

Analysis was conducted using with SPSS (Statistical Product and Service Solution), version 11.0. Multiple comparison tests (least significant difference, LSD) were used to detect differences in mycelial rate, sporulation and pathogennicity.

RESULTS

Sensitivity of *C.cassiicola* to carbendazim and diethefencarb

A total of 159 isolates of *C.cassiicola* were tested for their sensitivity to carbendazim and diethofencarb. Among these, 146 isolates were highly–resistant to carbendazim with MIC values higher than 100 µg/ml (Table 1). The frequency distribution of carbendazim resistant isolates was up to 91.8% (Fig.1), while 13 isolates were sensitivite to carbendazim with MIC values less than 10 µg/ml. The isolates whose MIC values between 10 and 100 µg/ml were not found (Table 1). 123 isolates of *C.cassiicola* resistant to carbendazim were more sensitive to diethofencarb, negative cross-resistance between carbendazim and diethofencarb was observed in *C.cassiicola*. The frequency distribution of diethofencarb-sensitive isolates was up to 84.2% (Fig.2), but 24 isolates were highly resistant to both carbendazim and diethofencarb and the frequency of double-resistant isolate was 14.5% (Table 1). The isolates collected in 2007 from rubber and Jasmine were all

Table 1. Sensitivity of *C.cassiicola* to carbendazim and diethofencarb

Year of isolation	Host	Location	Total no.of isolates	No. of isolates		
				M^{Sa}	M^{HRa}	$M^{HR}N^{HRa}$
2005-2011	Cucumber	Zhejiang Liaoning Hebei Beijing Shandong Hainan Heilongjiang Shanxi Neimenggu	108	0	98	10
2007-2011	Tomato	Beijing Hainan	14	3	7	4
2008-2009	Eggplant	Shandong Liaoning	9	0	5	4
2007-2011	Balsam pear	Shandong Liaoning Hebei	6	0	6	0
2009-2010	Cowpea	Liaoning Hebei Hainan	7	0	5	2
2007-2010	Kidney bean	Liaoning Hebei Hainan	4	0	1	3
2007	Rubber	HainanYunnan	9	9	0	0
2010	Pepper	Hainan	1	0	1	0
2007	Jasmine	Beijing	1	1	0	0

[a]M^{S}=carbendazim–sensitive strains; M^{HR}=highly carbendazim–resistant strains; $M^{HR}N^{HR}$= highly carbendazim and diethofencarb–resistant strains.

sensitive to carbendazim, while isolates collected in 2005–2011 from cucumber were all resistant to carbendazim. The isolates from other hosts were also have carbendazim–resistant or double–resistant isolates.

Fitness of resistant isolates

Carbendazim–resistant and double–resistant isolates grew normally on PSA without carbendazim or diethofencarb and there was no significant difference between resistant–isolates and their wild–type isolates. A similar situation was observed for sporulation and phytogenicity

Molecular analysis of the β-tubulin gene in *C.cassiicola*

To determine whether point mutation in the deduced amino acid sequences were related to resistance carbendazim and carbendazim and diethofencarb, the nucleotide sequence of β-tubulin were analyzed among M^S, M^{HR} and $M^{HR}N^{HR}$ isolates.

A 1122bp segment of the β-tubulin gene was amplified, which covered the 50, 167, 198, 200, 240 amino acid codon positions using the forward primer β-tubf1–F and reverse primer β-tubf1–R (GenBank accession no. JQ965171–965183). In the 13 isolates analyzed, resistance was correlated with a single nucleotide substitutions, conferring changes amino acid 198 or 200. All of the *C.cassiicola* isolates highly resistant to carbendazim (M^{HR}) had a single mutation (from GAG to GCG) in the codon corresponding to amino acid residue 198, which resulted in the substitution

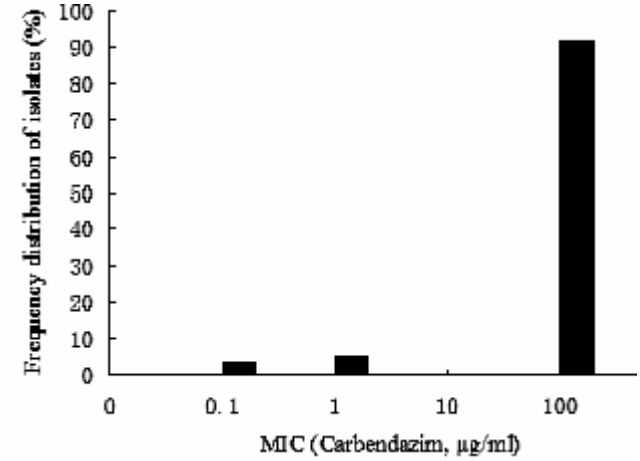
Table 2. Growth rate, sporulation, pathogenicity of wildtype, carbendazim–resistant and double resistant–isolates of *C.cassiicola*

Isolate	Sensitivity phenotype	Colony diameter(mm)	Sporulation ($\times10^5$)	Pathogenicity
FQ07091401	M^S	55.17a[a]	1.78a	79.5 0a
FQ10020102	M^S	50.83a	2.50a	82.80a
FQ0804160201	M^{HR}	53.83a	2.33a	84.27a
FQ10013103	M^{HR}	53.00a	2.89a	81.40a
FQ1108070801	M^{HR}	50.17a	1.89a	84.53a
FQ10222202	$M^{HR}N^{HR}$	52.00a	2.83a	85.40a
FQ1108070802	$M^{HR}N^{HR}$	59.83a	2.9a	83.70a

[a]Figures followed by the same letter within a column were not significantly different with LSD (least significant different) test at p=0.05

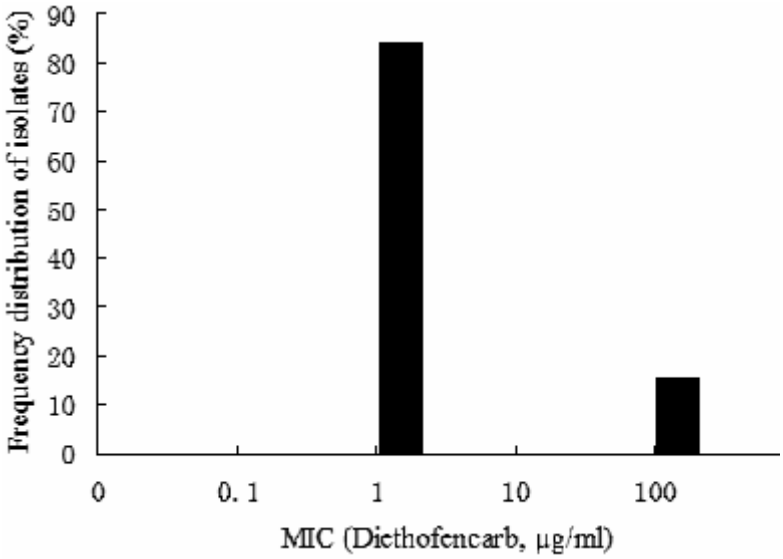
Table 3. Response of *C.cassiicola* to carbendazim and diethofencarb and deduced amino acid substitution in the sequence of β-tubulin gene at codons 198 and 200

Isolate	Host	Location	Year isolated	Sensitivity Carbendazim	Diethofencarb	Sequence in codon (amin acid)	
HCCHN42	Rubber	Hainan	2007	S	HR	198	200
MLH1	Jasmine	Beijing	2007	S	HR	GAG(Glu)	TTC(Phe)
FQ07091401	Tomato	Beijing	2007	S	HR	GAG(Glu)	TTC(Phe)
HG08032103	Cucumber	Shandong	2008	HR	S	GAG(Glu)	TTC(Phe)
HG08120404	Cucumber	Hebei	2008	HR	S	GCG(Ala)	TTC(Phe)
HG09031509	Cucumber	Neimenggu	2009	HR	S	GCG(Ala)	TTC(Phe)
HG08122201	Cucumber	Liaoning	2008	HR	S	GCG(Ala)	TTC(Phe)
HG07010804	Cucumber	Shandong	2007	HR	S	GCG(Ala)	TTC(Phe)
QZ09012006	Eggplant	Shandong	2009	HR	HR	AAG(Lys)	TTC(Phe)
HG09102301	Cucumber	Liaoning	2009	HR	HR	AAG(Lys)	TTC(Phe)
HG10110702	Cucumber	Shandong	2010	HR	HR	AAG(Lys)	TTC(Phe)
HG09031508	Cucumber	Neimenggu	2009	HR	HR	GAG(Glu)	TAC(Tyr)
CD10013103	Kidney bean	Hainan	2010	HR	HR	GAG(Glu)	TAC(Tyr)



Caption: Frequency distribution of carbendazim–sensitive and resistant isolates

Fig. 1. Frequency distribution of carbendazim



Caption: Frequency distribution of diethofencarb–sensitive and resistant isolates from carbendazim resistant isolates

Fig. 2. Frequency distribution of diethofencarb

of glutamic acid by alanine. Three isolates highly resistant to carbendazim and diethofencarb ($M^{HR}N^{HR}$) had a single mutation (from GAG to AAG) in the codon corresponding to amino acid residue 198, which resulted in the substitution of glutamic acid by lysine. Two isolates highly resistant to carbendazim and diethofencarb ($M^{HR}N^{HR}$) had a single mutation (from TTC to TAC) in the codon corresponding to amino acid residue 200, which resulted in the substitution of phenylalanine by tyrosine (Table 3). No additional mutations were found.

DISCUSSION

This is the first study of the sensitivity of *C.cassiicola* to carbendazim and diethofencarb in China. Although there are no registered fungicides for Corynespora leaf spot of cucumber in China, carbendazim–resistant isolates were found and the

frequency of resistant–isolates was surprisingly high (100%), Similar results also appeared in tomato. Carbendazim was applied for control grey mould of cucumber and tomato, the natural populations of *C.cassiicola* were also subjected to selection by carbendazim. The high selection pressure of carbendazim, combine with heavy occurrence of the disease, may be important reasons for the high frequency of carbendazim–resistant isolates of *C.cassiicola*.

The results showed carbendazim–resistant isolates were widely distributed in field populations, so carbendazim cannot be used to control Corynespora leaf spot of cucumber in China. Carbendazim and diethofencarb double–resistant isolates were also detected and this is the first report of *C. cassiicola* isolates that are highly resistant to both carbendazim and diethofencarb in China. It has been detected in Japan previously[13]. It is thought that *C. cassiicola* is a highrisk pathogen for fungicide resistance development[14], the frequency of double–resistant isolates was up to 14.5% , and their fitness haven't significantly reduced, so diethofencarb should not be used to control *C.cassiicola* in China without reasonable resistance management strategies.

Other fungicides, such as dicarboximides, Q_0I fungicides and bascalid were very effective for controlling this disease[8, 13, 14, 15], so it is necessary to determine the sensitivity of *C.cassiicola* to these fungicides. Especially bascalid, in Japan, it has been registered commercially for the control of corynespora leaf spot of cucumber in 2006. However, In China, bascalid was officially registered for control of grey mould of cucumber in December 2008, The influence of controlling grey mould to the development of bascalid–resistant isolates of *C.cassiicola* in natural populations was to be concerned.

Corynespora leaf spot of pepper is a new disease in China, it has been reported in Korea and Japan repectively and leaded to massive losses in pepper production[16, 17]. Interestingly, the planting site didn't appliy carbendazim or other benzimidazole fungicides, but carbendazim resistant–isolate was detected, similar result was also found in *C.cassiicola* resistant to boscalid in Japan[18], the reason is yet unclear. the spore of carbendazim–resistant fungal from other hosts infect the pepper may be a possibile reason. In several monitoring studies, long–diatance dispersal of resistant fungal conidia by wind has been proposed[19, 20] and was demonstrated in Q_0I resistance of *Mycospbaerella graminicola*[21]. It has been reported that some isolates of *C.cassiicola* show virulence on a wide range of hosts, whereas others exhibit host specifity[22, 23, 24, 25, 26, 27]. But there is little research about host specificity of *C.cassiicola* in China, the cross–infectivity of *C.cassiicola* between pepper and other hosts in China needs a further study.

Nine isolates collected in 2007 from rubber were sensitive to carbendazim. In 2006 *C.cassiicola* was identified on *Hevea* rubber in Hainan and Yunnan provinces, China[28]. By the end of 2007, more than 200 ha of *Hevea* rubber were infected and their incidence rate reached 94%. The most economical way of controlling this disease is the use of resistant cultivars, but most of *Hevea* rubber clones are highly susceptible in China[29]. So chemical control is an important method for managing this disease. As the report by IRRDB (The International Rubber Research and Development Board), benomyl, which is belongs to benzimidazole fungicides, was considered the most effective fungicide in field. If it use in this disease control, Monitoring the development of resistance is necessary.

In this study ,we observed only one resistance level (high resistance) to carbendazim. However, both moderate and high resistant isolates of *C.cassiicola* were observed in field isolates in Japan[15]. Whether carbendazim–moderate isolates existed in field need a further study. 3 sensitivity phenotypes (M^S, M^{HR} and $M^{HR}N^{HR}$) to carbendazim and diethofencarb were found in this study. Comparison of the nucleotide sequence of â–tubulin gene among 3 phenotypes revealed that M^{HR} isolates of *C.cassiicola* had only a single mutation of GAG in M^S isolates to GCG, which caused a change of glutamic acid (GAG) to alanine (GCG) at codon position 198 of â–tubulin gene. Glutamic acid (GAG) at codon 198 was changed to lysine and phenylalanine (TTC) at 200 was changed to tyrosine (TAC) in $M^{HR}N^{HR}$ isolates of *C.cassiicola*. However, the mutation Phe200Tyr generally result in moderate resistance to benzimidazole fungicides and insensitivity to diethofencarb in other fungus[9, 10, 30], additional resistance mechanism may be exist.

These finding have reported from other phytopathogeni fungi[31, 32, 33], which raises the possibility of development a rapid screen to distinguished 3 sensitivity phenotypes isolates. Such screen have been examined for *Botrytis cinerea* and could be useful to growers in making informed management decision[34, 35].

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