Multiple Fungicide Resistance in *Botrytis cinerea* from Greenhouse Strawberries in Hubei Province, China

F. Fan, Key Lab of Horticultural Plant Biology, Ministry of Education, and College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, China; **M. S. Hamada**, College of Plant Science and Technology, Huazhong Agricultural University, and Pesticides Department, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt; **N. Li** and **G. Q. Li**, College of Plant Science and Technology and the Key Lab of Crop Disease Monitoring & Safety Control in Hubei Province, Huazhong Agricultural University; and **C. X. Luo**, Key Lab of Horticultural Plant Biology, Ministry of Education, and College of Plant Science and Technology, Huazhong Agricultural University

Abstract

Two hundred and forty isolates of *Botrytis cinerea* were collected during the early summer of 2012 and 2013 from strawberry greenhouses in 10 locations in Hubei Province and examined for sensitivity to five fungicides, most of which were commonly used to control this fungus. High frequency of resistance to carbendazim (Car, 63.63%) and cyprodinil (Cyp, 42.42%) was detected. Boscalid-resistant (Bos^R) isolates were detected for the first time in China, whereas no fludioxonil-resistant isolates were identified. Dual resistance to carbendazim and diethofencarb (Die) was also detected. There were six phenotypes of resistance profile (i.e., Car^RDie^SBos^SCyp^S, Car^RDie^RBos^SCyp^S, Car^RDie^SBos^SCyp^R, Car^RDie^SBos^RCyp^S, Car^RDie^RBos^SCyp^R, and Car^RDie^SBos^RCyp^R). Car^RDie^SBos^SCyp^S and Car^RDie^SBos^SCyp^R were the most common phenotypes, occurring at eight and seven locations, respectively. After 10 successive transfers on fungicide-free potato dextrose agar, tested

resistant isolates retained levels of resistance similar to or comparative with the initial generation, indicating the stability of these resistances. Fitness evaluations based on investigation of mycelial growth, osmotic sensitivity, sporulation in vitro and in vivo, and virulence revealed the uncompromising fitness in resistant isolates, except that decreased virulence was observed in Bos^R isolates. The molecular basis of carbendazim, diethofencarb, and boscalid resistance was investigated. Results showed that all 13 sequenced carbendazim-resistant isolates harbored the mutation E198V or E198A in the β -tubulin gene and the five isolates with dual resistance to carbendazim and diethofencarb showed the mutation E198K in the same gene. Bos^R isolates possessed the H272R mutation in succinate dehydrogenase subunit B gene. The results achieved in this study challenge the current management strategies for *B. cinerea*, which largely depend on applications of these fungicides.

Strawberry is an important commercial fruit crop, widely grown in all temperate regions in the world. In 2012, China was the largest strawberry-growing country in the world, where the area of strawberry crops was over 100,000 ha and more than two million tons of strawberry fruit were produced (Xiang et al. 2015). Among all the provinces in China, Hubei Province was in the top six, where about 11,000 ha of strawberry plants were cultivated (Xiang et al. 2015). However, the temperate climatic condition is also suitable for the development of many diseases. Among fungal diseases, gray mold, caused by *Botrytis cinerea* Pers. (teleomorph: *Botryotinia fuckeliana*), is one of the most economically important diseases both pre- and postharvest (Fernández-Ortuño et al. 2012).

For gray mold management, benzimidazoles (MBC), N-phenylcarbamates (NPC), dicarboximides, quinone outside inhibitors, succinate dehydrogenase inhibitors (SDHI), anilinopyrimidines (AP), and phenylpyrroles (PP) are currently available. However, the efficacy of fungicides has been decreased by the emergence of resistant isolates. *Botrytis cinerea* belongs to a classical high-risk pathogen for the development of fungicide resistance, due to its high genetic variability, abundant sporulation, polycyclic nature, and the high number of sprays required for its successful control (Brent et al. 1998; Leroux et al. 2002). In recent decades, resistance of *B. cinerea* to these fungicides has been reported in different countries after their introduction for the control of gray mold (Bardas et al. 2010; Baroffio et al. 2003; Sun et al. 2010; Weber 2011).

MBC fungicides such as carbendazim were the first systemic single-site fungicides that were introduced into agriculture in the 1970s (Walker et al. 2013). The fungicidal activity of MBC results from their inhibition of germ-tube elongation and mycelial growth by binding to β -tubulin, preventing microtubule assembly (Leroux et al. 1999). In

B. cinerea, resistance was selected soon after the introduction of fungicides with this mode of action (Bolton 1976; Tripathi and Schlosser 1982). The resistance was generally conferred by mutations of the β -tubulin (*TUB2*) gene at codon 198, which altered from glutamic acid to alanine (E198A), valine (E198V), or lysine (E198K) (Leroux et al. 2002; Malandrakis et al. 2011; Yarden and Katan 1993).

The NPC fungicide diethofencarb also represents an antimicrotubule fungicide. This fungicide was generally used as a mixture with carbendazim to control *B. cinerea* because it frequently reveals negative cross resistance with MBC fungicides (Leroux et al. 1999; Zhang et al. 2009b). However, the intensive application of the mixture resulted in the development of dual resistance to carbendazim and diethofencarb in different countries (Elad et al. 1992; Sun et al. 2010). This dual resistance was generally involved with the E198K or F200Y mutation of the *TUB2* gene (Malandrakis et al. 2011; Yarden and Katan 1993).

Boscalid, registered as botryticide in recent years, is an SDHI fungicide. The mode of action is to prevent mitochondrial respiration by inhibiting the activity of mitochondrial respiratory complex II, also known as succinate dehydrogenase (SDH), in the mitochondrial respiratory chain (Kim and Xiao 2011). SDH comprises four subunits (SdhA, SdhB, SdhC, and SdhD) and plays a crucial role in the tricarboxylic cycle. The inhibition of this enzyme leads to a block of the cell energy cycle, thereby leading to cell death (Horsefield et al. 2006). The site-specific mode of action of this fungicide enables the fungus to develop resistance easily, based on very few genetic modifications. Mutations in the SdhB subunit resulting in the substitution from proline to leucine, phenylalanine, or threonine at codon 225 (P225L, P225F, or P225T); from histidine to tyrosine, arginine, or leucine at codon 272 (H272Y, H272R, or H272L); or from asparagine to isoleucine at codon 230 (N230I) were frequently detected in laboratory mutants or resistant field isolates and have been confirmed to confer resistance to SDHI in *B. cinerea* (Fernández-Ortuño et al. 2012; Lalève et al. 2014b). In addition, the substitution from histidine to arginine at codon 132 (H132R) in the SdhD subunit was detected in field isolates with resistance to boscalid (Leroux et al. 2010).

AP fungicides such as pyrimethanil and cyprodinil were introduced to control *B. cinerea* in recent decades. It is suggested that AP inhibit

Corresponding author: C. X. Luo; E-mail: cxluo@mail.hzau.edu.cn

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the biosynthesis of methionine and other amino acids, and prevent the secretion of hydrolytic enzymes involved in the infection process (Fernández-Ortuño et al. 2013b; Masner et al. 1994). However, the exact mode of action of this fungicide has not been clarified (Fritz et al. 1997; Masner et al. 1994; Milling and Richardson 1995). Resistance to AP of *B. cinerea* from fields has been reported in many studies soon after its application (Bardas et al. 2010; Li et al. 2014a; Weber 2011; Zhang et al. 2009a), indicating that there is a high risk for the development of resistance to this class of fungicides.

Fludioxonil is one of the PP fungicides and is very effective against *B. cinerea* on many crops (Li et al. 2014b). Like AP, the primary mode of action of this fungicide remains unknown as well. It has been hypothesized that PP inhibit the transport-associated phosphorylation of glucose and interfere with the osmotic signal transduction pathway by inhibiting a protein kinase (PK-III) (Fernández-Ortuño et al. 2013b; Jespers and De Waard 1995; Pillonel and Meyer 1997). Resistant field isolates have been described in recent years in France, Germany, and the United States (Fernández-Ortuño et al. 2013a,b, 2014b,c; Kretschmer et al. 2009). The mechanisms of this resistance may be related to the overexpression of ATP-binding cassette transporter gene *BcatrB* and major facilitator superfamily transporter gene *MfsM2*, and mutations in the transcription factor *Mrr1* (Kretschmer et al. 2009; Li et al. 2014b; Vermeulen et al. 2001).

In Hubei, MBC, NPC, SDHI, and AP fungicides have been routinely applied to control gray mold. In recent years, efficacy of some fungicides has decreased. The objectives of this study were to (i) determine the resistance profiles of *B. cinerea* from strawberry greenhouses in Hubei, China to carbendazim, diethofencarb, boscalid, fludioxonil, and cyprodinil; (ii) evaluate the fitness of resistant isolates in vitro and in vivo; and (iii) detect the point mutations in corresponding target genes. The information obtained in this study is crucial for designing new strategies to manage fungicide resistance that has become highly developed in *B. cinerea* populations in Hubei, China.

Materials and Methods

Fungicides. Technical-grade carbendazim (92.0% active ingredient [a.i.]; Hubei Jianyuan Chemical Co., Ltd.) was dissolved in lactic acid to obtain a stock solution of 10,000 µg a.i./ml. Technical-grade diethofencarb (95.4% a.i.; Jiangsu Lanfeng Biochemical Co., Ltd.), cyprodinil (98.1% a.i.; Hubei Kangbaotai Fine-chemicals Co., Ltd.), boscalid (97.0% a.i.; Hubei Kangbaotai Fine-chemicals Co., Ltd.), and fludioxonil (97.0% a.i.; Hubei Jianyuan Chemical Co., Ltd.) were dissolved in acetone to obtain stock solutions of 5,000, 2,000, 10,000, and 200 µg a.i./ml, respectively. Stock solutions were kept at 4°C in

darkness until use. In order to evaluate the control efficacy for resistant isolates, commercial products of different fungicides were applied; for example, Guoguang (50.0% WP of carbendazim; Sichuang Guoguang Agrochemical Co., Ltd.), Gold Wanmeiling (25.0% WP of carbendazim and 25.0% diethofencarb; Jiangsu Lanfeng Biochemical Co., Ltd.), Rongya (50.0% WPG of cyprodinil; Jixi Nonghua Biotechnology Co., Ltd.), and Cantus (50.0% WPG of boscalid; BASF Co., Ltd.).

Collection of *B. cinerea* isolates. During 2012 and 2013, 240 single-spore isolates of *B. cinerea* were isolated from diseased strawberry fruit or leaves from different greenhouses at various locations in Hubei (Table 1). Among these locations, the shortest linear distance was around 50 km between Xiantao and Qianjiang. The longest linear distance was over 400 km between Shiyan and Xianning. The average linear distance was over 100 km between two cities. Isolates were preserved on dried filter paper for long-term storage, as described before (Luo et al. 2002). In brief, the isolates were inoculated on potato dextrose agar (PDA; 200 ml of juice from 200 g of potato, 20 g of dextrose, and 15 g of agar per liter) with filter-paper disks for 5 days. Then, disks with mycelium were collected, desiccated, and stored at -20°C. All 240 isolates were identified as *B. cinerea* by both morphological observation and polymerase chain reaction (PCR)-based assay, in which the *B. cinerea*-specific 327-bp fragment was amplified using the primer pair Bc-f/Bc-r (CAG GAA ACA CTT TTG GGG ATA/GAG GGA CAA GAA AAT CGA CTA A) (Fan et al. 2015).

Determination of fungicide resistance in *B. cinerea*. To distinguish resistant isolates from sensitive ones, the discriminatory concentration (a concentration that fully inhibits mycelial growth of the sensitive isolates) was used. According to previous studies, the discriminatory concentrations were 10, 5, 0.4, 75, and 4 µg/ml for carbendazim, diethofencarb, fludioxonil, boscalid, and cyprodinil, respectively (Fernández-Ortuño et al. 2014a; Sun et al. 2010). The 1.0% malt extract agar medium was used for testing resistance to carbendazim, diethofencarb, and fludioxonil while 1.0% yeast bacto acetate agar (YBA) medium was used for boscalid resistance and Czapek-Dox agar medium (CzA) was used for cyprodinil resistance (Fernández-Ortuño et al. 2014a). Mycelial plugs (5 mm in diameter) were cut with a cork borer from the margin of a 3-day-old colony of each isolate and placed upside down onto the media amended with fungicide. The colony diameter was measured after 3 days at 22°C. For each fungicide, isolates that were able to grow on the amended media were considered to be resistant isolates, while isolates unable to grow on the amended media were considered to be sensitive isolates. For sensitivity to diethofencarb, only isolates resistant to carbendazim were tested because the isolates sensitive to carbendazim are naturally resistant to diethofencarb. The experiment had three replicates.

Evaluation of the stability of resistance in vitro. An adjusted method based on a previous study was adopted to evaluate the stability of resistance in vitro (Kim and Xiao 2011). Two isolates resistant to boscalid (Bos^R), three isolates resistant to carbendazim (Car^R), and six isolates resistant to carbendazim, diethofencarb, and cyprodinil (Car^RDie^RCyp^R) were randomly selected. These isolates were subcultured every 4 days for 10 generations on fungicide-free PDA plates at 22°C in the dark. Sensitivity of selected isolates to carbendazim and diethofencarb was determined in a mycelial growth assay on PDA amended with each fungicide at 0, 0.3, 1, 3, 10, 30, and 100 µg a.i./ml. Sensitivity to boscalid and cyprodinil was determined on YBA and CzA amended with each fungicide at 0, 0.3, 1, 3, 10, 30, and 100 µg a.i./ml, respectively. The sensitivity test was conducted before the first and after the last transfer and three replicate plates were used for each isolate. The colony diameters were determined after 3 days of incubation at 22°C by calculating the mean of two perpendicular colony diameters. The percentage of mycelial growth inhibition was calculated according to the formula percent inhibition = (1 - mean colony diameter on treated plates/mean colony diameter on nontreated plates) × 100%. The assessed concentration at which mycelial growth is inhibited by 50% (EC₅₀ value) was calculated by regressing percentage of mycelial growth inhibitions against the log₁₀ of fungicide concentrations. The experiment was conducted twice.

Measurement of fungal mycelial growth and sporulation in vitro. In all, 6 sensitive isolates and 11 resistant isolates used in stability of

Table 1. Frequency of resistance in *Botrytis cinerea* collected from greenhouses of strawberry in Hubei Province during 2012 and 2013

Location ^x	N ^y	Frequency of resistant isolates (%) ^w				
		Car	Die ^z	Cyp	Bos	Flu
EZ	18	0	nd	0	0	0
QJ	23	17.39	0	0	0	0
SY	11	45.45	0	27.27	0	0
WH	33	63.63	9.52	42.42	3.03	0
XT	24	33.33	12.5	12.50	0	0
XN	14	21.43	0	7.14	0	0
XY	33	3.03	100	3.03	0	0
XG	31	41.94	23.08	19.35	0	0
YC	32	59.38	21.05	37.50	0	0
SZ	21	42.85	11.11	23.81	4.76	0
Total	240	34.59	14.46	18.75	0.83	0

^w Car = carbendazim, Die = diethofencarb, Cyp = cyprodinil, Bos = boscalid, and Flu = fludioxonil; nd = not detected.

^x EZ = Ezhou, QJ = Qianjiang, SY = Shiyan, WH = Wuhan, XT = Xiantao, XN = Xianning, XY = Xiangyang, XG = Xiaogan, YC = Yichang, and SZ = Suizhou.

^y Number of isolates.

^z Diethofencarb resistance was only tested when isolates were resistant to carbendazim.

resistance evaluation were used. The mycelial growth of each isolate was measured on fungicide-free PDA plates. Four plates of each isolate were inoculated with mycelial plugs and incubated at 22°C for 3 days. The colony diameters were then measured at two perpendicular directions. The experiment was conducted twice.

To investigate sporulation of each isolate, PDA plates inoculated with mycelial plugs were incubated at 22°C for 3 weeks in the dark. Then, each plate was rinsed with 3 ml of sterile water, and the conidial suspension was filtered through one layer of lens-cleaning paper. The conidia concentration was determined with a hemacytometer and expressed as number of conidia per square centimeter of the PDA culture. Three droplets of the suspension were counted for each plate and four plates were used for each isolate. The experiment was conducted twice.

Determination of osmotic sensitivity. Osmotic sensitivity was determined by comparing the radial growth of isolates on PDA with that on PDA amended with 8.0% NaCl. Colony diameters were measured after 3 days of incubation at 22°C. The percentage of mycelial growth inhibition was calculated as described in the sensitivity test. Four replicate plates were used for each isolate, and the experiment was conducted twice.

Evaluation of fungal virulence and sporulation in vivo. The virulence of the 17 isolates with different resistance phenotypes was determined on strawberry leaves. Leaves with similar size and maturity were washed with dish detergent, immersed in NaClO at 600 mg/liter for 5 min, then rinsed with tap water to remove the chemical residue. Leaves were put on 3-cm-diameter plastic rings in plastic boxes with tissue soaked with sterile water at the bottom. After leaves were air dried, each leaf was punctured with a blood lancet (Yangzhou Shuangling Medical Appliance Co., Ltd.) in the middle, after which a 5-by-2-mm plug taken from the periphery of a 3-day-old colony on PDA was put on the wound. After inoculation, the plastic boxes were covered with cling film to maintain high humidity. Lesion diameter was recorded after 5 days of incubation at 22°C in darkness. Six replicate leaves were applied to each isolate and the experiment was conducted twice.

After the measurement of lesion diameter, strawberry leaves were put back for an additional incubation to induce sporulation. After 14 days at 22°C with a 12-h photoperiod, leaf discs (about 0.2 cm²) with sporulating lesions were collected with the aid of a 5-mm-diameter cork borer. One disc was obtained from each leaf and six leaves were applied for each isolate. Each leaf disc obtained was placed in 1 ml of distilled water in an Eppendorf tube, and vortexed to detach the conidia from the lesions. The spore concentration was determined with the aid of hemacytometer. Four replicate droplets were measured for each tube. The sporulation was expressed as number of conidia per square centimeter of diseased strawberry leaf surface.

Evaluation of control efficacy of fungicides to resistant isolates. Three sensitive isolates, two Bos^R isolates, and three Car^RDie^RCyp^R isolates were randomly selected to test fungicide efficacy. Before inoculation, the leaves prepared as described above were treated with the highest recommended rate of Guoguang at 1.7 g/liter, Gold Wanmeiling at 1.7 g/liter, Rongya at 1.7 g/liter, or Cantus at 1.5 g/liter. The leaves sprayed with distilled water were used as control. The leaves were dried off in the air for 3 h, then inoculated as described above and incubated at 22°C for 5 days, after which lesion diameter was measured. Control efficacy was expressed as the percentage of the mean lesion diameter of the control minus the mean diameter of the treatment divided by the mean diameter of the control. This experiment was performed twice with five leaves per treatment.

Sequencing of the *TUB2* gene from Car^R isolates and *SdhB* gene from Bos^R isolates. Thirteen Car^R isolates, five isolates with dual resistance to carbendazim and diethofencarb, and two Bos^R isolates were analyzed. Two carbendazim-sensitive isolates and one boscalid-sensitive isolate were used as control. Mycelia were collected from 3-day-old colonies on PDA and genomic DNA was extracted and purified according to a previous method (Chi et al. 2009). The partial sequence of the *TUB2* gene was amplified with primer pair TubA F/R (GAT CTC CAA CTT GAG CGT A/TGG AAA CCT TAC CAC GGC TA) designed using Oligo7 software (Molecular Biology Insights Inc.). The partial *SdhB* gene was amplified with the primer pair IpBcBeg/IpBcEnd2 (CCA CTC CTC CAT AAT GGC TGC TCT

CCG C/CTC ATC AAG CCC CCT CAT TGA TAT C) described previously (Leroux et al. 2010). PCR products were checked by electrophoresis in a 1.0% agarose gel in 1× Tris-borate-EDTA buffer, and sequenced at Wuhan Tianyi Huiyuan Biotechnology Co., Ltd.

Data analysis. Fitness component data were subjected to one-way analysis of variance. Mean values among different groups were compared using a least significant difference test. Percentage data were arcsine transformed and sporulation data were logarithm transformed prior to analysis. The statistical analysis tests were performed by SPSS (version 19.0; IBM Corporation).

Results

The frequency of fungicide resistance at 10 locations in Hubei Province. Sensitivity to different fungicides was assayed for 240 isolates of *B. cinerea* from greenhouse strawberry plants at 10 different locations in Hubei Province. As a result, isolates with resistance to carbendazim were detected from almost all the sampling sites except Ezhou (EZ), with the frequency of resistance ranging from 3.03% in Xiangyang (XY) to 63.63% in Wuhan (WH) (Table 1). The dual resistance to carbendazim and diethofencarb occurred at most of the locations, except Qianjiang (QJ), Shiyan (SY), and Xianning (XN). Isolates with resistance to cyprodinil were also detected from most of the tested locations, except EZ and QJ. The frequency ranged from 3.03% in XY to 42.42% in WH. Isolates with resistance to boscalid were only found in WH and Suizhou (SZ), with frequencies of 3.03 and 4.76%, respectively. No isolates with fludioxonil resistance were detected (Table 1).

The frequency of different resistance phenotypes. Altogether, six resistance phenotypes (Car^RDie^SBos^SCyp^S, Car^RDie^RBos^SCyp^S, Car^RDie^SBos^SCyp^R, Car^RDie^SBos^RCyp^S, Car^RDie^RBos^SCyp^R, and Car^RDie^SBos^RCyp^R, where superscripts R and S indicate resistant and sensitive, respectively) were detected in the 240 isolates. Car^RDie^SBos^SCyp^S was the most common phenotype, occurring at eight tested locations. Car^RDie^SBos^SCyp^R was the second most common phenotype, detected from seven locations. Car^RDie^RBos^SCyp^S and Car^RDie^RBos^SCyp^R were found at three and four tested locations, respectively, while Car^RDie^SBos^RCyp^S and Car^RDie^SBos^RCyp^R were only detected at WH and SZ, respectively.

Stability of the resistant isolates. For carbendazim, the EC₅₀ values of most tested resistant isolates were slightly increased, although some of them were decreased after 10 successive transfers on fungicide-free PDA. For example, the EC₅₀ values of isolates HBStr-133, HBStr-298, and HBStr-291 decreased from 68.77, 89.25, and 56.15 to 37.34, 51.16, and 49.78, respectively, indicating that they still maintained the comparative resistance to carbendazim. Similar results were observed in cyprodinil resistance; four of the six isolates retained a level of resistance (EC₅₀ values >100) as high as their initial generation. Although the resistance level of the other two isolates increased (EC₅₀ value from 38.7 to 48.77 in HBStr-574) or decreased (from 45.68 to 34.25 in HBStr-133) a little, they still remained resistant. All the isolates with dual resistance to carbendazim and diethofencarb as well as Bos^R isolates retained the same level of sensitivity (EC₅₀ values >100) to corresponding fungicides as their initial generation.

Uncompromising fitness of resistant isolates. Apparent variability of mycelial growth, sporulation in vitro and in vivo, osmotic sensitivity, and virulence were detected among isolates from different groups, or even within the same sensitivity group. However, uncompromising fitness was observed in resistant isolates when different sensitivity groups were compared, except that the decreased virulence was observed in Bos^R isolates. The mean mycelial growth of the Car^R isolates, Bos^R isolates, and Car^RDie^RCyp^R isolates was not significantly different from that of the sensitive isolates ($P > 0.05$). Similarly, the mean value of sporulation in vitro of the Car^R isolates, Bos^R isolates, and Car^RDie^RCyp^R isolates was not significantly different from that of the sensitive isolates ($P > 0.05$; Table 2). For osmotic sensitivity, the mean percent inhibition of the Car^R isolates, Bos^R isolates, and Car^RDie^RCyp^R isolates was also not significantly different from that of the sensitive isolates ($P > 0.05$; Table 2). All of the isolates, including sensitive, Car^R, Bos^R, and Car^RDie^RCyp^R isolates, caused severe rot on strawberry leaves. The mean lesions of the Car^R isolates and

Car^RDie^RCyp^R isolates were not significantly different from the sensitive isolates ($P > 0.05$) but the mean lesion size of the Bos^R isolates was significantly smaller than that of the sensitive isolates ($P < 0.05$). When the ability of sporulation in vivo was compared, there was no significant difference between the resistant and sensitive isolates ($P > 0.05$; Table 2).

Fungicide control efficacy for resistant isolates. Two Bos^R isolates, three Car^RDie^RCyp^R isolates, as well as three sensitive isolates were chosen for assays of fungicide control efficacy on detached leaves. Guoguang (carbendazim) and Gold Wanmeiling (carbendazim and diethofencarb) could inhibit the sensitive isolates completely. Some sensitive isolates could cause diseases even with the application of Rongya (cyprodinil) or Cantus (boscalid) but most of the control efficacy was near 90% (Table 3). In contrast, the highest field rate of Guoguang, Gold Wanmeiling, or Rongya could not control the resistant isolates, and the disease incidence was 100%. Compared with the sensitive isolates, most of the control efficiency was <0%, except for isolate HBStr-155. For isolate HBStr-155, the control efficacy of carbendazim, carbendazim and diethofencarb, and cyprodinil was 8.93, 10.90, and 15.49%,

respectively. The field rate of Cantus also could not control the resistant isolates, and the control efficiency for two resistant isolates was only 21.94 and 41.25%, respectively (Table 3).

Resistance was conferred by point mutations in target genes. Single-nucleotide changes at codon 198 were detected in the partial *TUB2* gene from 13 Car^R isolates. Ten isolates showed the mutation from GAG to GTG (E198V), and the other three showed the mutation from GAG to GCG (E198A). Interestingly, mutation from GAG to AAG (E198K) was detected in five isolates with dual resistance to carbendazim and diethofencarb.

Sequence analysis of the partial *SdhB* gene from two Bos^R isolates revealed a single nucleotide change at codon 272. Both of them showed the mutation from CAC to CGC, changing the amino acid from histidine to arginine (H272R).

Discussion

Development of fungicide resistance in gray mold pathogen *B. cinerea* is a real problem and challenge for the strawberry producers in

Table 2. Determination of fitness of *Botrytis cinerea* isolates with different resistant phenotypes^w

Isolates	Phenotypes ^x	In vitro experiments			In vivo experiments	
		MG (cm)	OS ^y	Spor ($\times 10^5/\text{cm}^2$) ^z	LS (cm)	Spor ($\times 10^5/\text{cm}^2$) ^z
HBStr-104	Car ^S Die ^R Bos ^S Cyp ^S	7.01 b	77.69 d	3.26 b	2.10 ab	17.51 a
HBStr-124	Car ^S Die ^R Bos ^S Cyp ^S	7.22 ab	86.63 bc	1.98 bc	2.43 a	17.56 a
HBStr-354	Car ^S Die ^R Bos ^S Cyp ^S	5.64 d	76.96 d	0.24 d	1.73 b	9.34 a
HBStr-489	Car ^S Die ^R Bos ^S Cyp ^S	6.91 b	78.14 d	0.39 cd	1.96 ab	12.31 a
HBStr-367	Car ^S Die ^R Bos ^S Cyp ^S	7.09 b	80.03 cd	2.36 bc	2.57 a	18.41 a
HBStr-556	Car ^S Die ^R Bos ^S Cyp ^S	6.77 b	77.83 d	17.89 a	2.02 ab	17.88 a
Mean	Car ^S Die ^R Bos ^S Cyp ^S	6.77	79.55	4.35	2.14	15.50
HBStr-114	Car ^R Die ^S Bos ^R Cyp ^S	6.36 c	86.31 bc	3.52 bc	1.48 b	14.64 a
HBStr-278	Car ^R Die ^S Bos ^R Cyp ^R	7.27 ab	78.26 d	0.68 c	0.74 c	11.09 a
Mean	Car ^R Die ^S Bos ^R Cyp ^R	6.82	82.29	2.10	1.11	12.87
HBStr-291	Car ^R Die ^S Bos ^S Cyp ^S	7.46 ab	77.52 d	0.00 f	2.37 a	0.95 b
HBStr-317	Car ^R Die ^S Bos ^S Cyp ^S	6.69 bc	87.68 b	7.22 ab	1.71 b	17.45 a
HBStr-491	Car ^R Die ^S Bos ^S Cyp ^S	6.76 bc	83.01 c	3.96 b	2.04 ab	18.73 a
Mean	Car ^R Die ^S Bos ^S Cyp ^S	6.97	82.74	3.73	2.04	12.38
HBStr-133	Car ^R Die ^R Bos ^S Cyp ^R	6.23 c	92.35 a	11.23 ab	1.67 b	10.61 a
HBStr-155	Car ^R Die ^R Bos ^S Cyp ^R	6.11 c	84.41 bc	1.64 bc	2.15 ab	7.16 a
HBStr-298	Car ^R Die ^R Bos ^S Cyp ^R	7.22 ab	80.65 cd	0.04 d	1.60 b	1.80 b
HBStr-301	Car ^R Die ^R Bos ^S Cyp ^R	6.93 b	82.25 cd	0.72 c	2.01 ab	11.99 a
HBStr-527	Car ^R Die ^R Bos ^S Cyp ^R	7.49 a	79.40 cd	0.98 bc	2.38 a	11.83 a
HBStr-574	Car ^R Die ^R Bos ^S Cyp ^R	6.83 b	85.82 bc	0.02 e	1.28 bc	13.21 b
Mean	Car ^R Die ^R Bos ^S Cyp ^R	6.80	84.15	2.44	1.85	9.43

^w MG = mycelial growth, OS = osmotic sensitivity, Spor = sporulation, and LS = lesion size. Data shown are the means of two independent experiments. Values followed by the same letter within a column are not significantly different according to the one-way analysis of variance with least significant difference test at $P = 0.05$.

^x Car = carbendazim, Die = diethofencarb, Bos = boscalid, and Cyp = cyprodinil; superscripts R and S indicate resistant and sensitive, respectively.

^y Percent inhibition of mycelial growth on potato dextrose agar amended with 8% NaCl relative to the unamended control. Data were arcsine-transformed before analysis.

^z Data were logarithm-transformed before analysis.

Table 3. Lesion size caused by resistant isolates and their control efficacy on detached leaves treated with formulated fungicides^w

Isolates	Phenotypes ^z	Disease incidence (%) ^x				Control efficacy (%) ^y			
		Car	Car + Die	Cyp	Bos	Car	Car + Die	Cyp	Bos
HBStr-104	Car ^S Die ^R Bos ^S Cyp ^S	0 b	0 b	100 a	20 b	100 a	100 a	77.40 a	95.73 a
HBStr-354	Car ^S Die ^R Bos ^S Cyp ^S	0 b	0 b	100 a	nd	100 a	100 a	85.88 a	nd
HBStr-489	Car ^S Die ^R Bos ^S Cyp ^S	0 b	0 b	100 a	40 b	100 a	100 a	88.41 a	95.26 a
HBStr-133	Car ^R Die ^R Bos ^S Cyp ^R	100 a	100 a	100 a	nd	32.70 b	-6.16 b	-18.09 c	nd
HBStr-155	Car ^R Die ^R Bos ^S Cyp ^R	100 a	100 a	100 a	nd	8.93 b	10.90 b	15.49 b	nd
HBStr-301	Car ^R Die ^R Bos ^S Cyp ^R	100 a	100 a	100 a	nd	-24.58 b	-3.51 b	13.47 c	nd
HBStr-114	Car ^R Die ^S Bos ^R Cyp ^S	nd	nd	nd	100 a	nd	nd	nd	41.25 b
HBStr-278	Car ^R Die ^S Bos ^R Cyp ^R	nd	nd	nd	100 a	nd	nd	nd	21.94 b

^w Data are the means of two independent experiments. Values followed by the same letter within a column are not significantly different according to the one-way analysis of variance with least significant difference test at $P = 0.05$; nd = not detected.

^x Disease incidence was expressed as the percentage of wounds infected compared with the total number of wounds inoculated.

^y Control efficacy was expressed as the percentage of the mean diameter of the control minus the mean diameter of the treatment divided by the mean diameter of the control.

^z Car = carbendazim, Die = diethofencarb, Bos = boscalid, and Cyp = cyprodinil; superscripts R and S indicate resistant and sensitive, respectively.

Hubei Province. In order to make an effective strategy to control this disease, it is necessary to investigate the resistance profile of *B. cinerea*. Therefore, this study was performed and results showed that fungicide resistance in *B. cinerea* from greenhouse strawberry plants was widespread in Hubei Province and the multiresistance phenotypes of *B. cinerea* have developed in this area of China.

High frequencies of resistance to carbendazim (up to 63.63%) were detected at some locations. The highly developed resistance might be a result of high selection pressure derived from the extensive use of the corresponding fungicide. Similar results were reported in eastern provinces of China such as Jiangsu, Zhejiang, and Shandong, where the frequency was 100% for carbendazim (Sun et al. 2010; Zhang et al. 2009b). Liu et al. (2016) observed very high frequencies (89%) of resistance for carbendazim and diethofencarb in the northern province of Henan in 2016. A similar observation was also recorded worldwide (Fernández-Ortuño et al. 2014a; Weber 2011). Although cyprodinil was introduced recently in China to control gray mold disease, high frequencies of cyprodinil resistance were detected in this study (WH 42.42% and Yichang 37.50%). This may be due to the extensive use of the other anilinopyrimidine fungicide pyrimethanil in Hubei Province during the last 10 years. A similar result was also observed in the strawberry fields in North and South Carolina, where 47% isolates collected were Cyp^R (Fernández-Ortuño et al. 2013b). It was reported that, when a single application of an AP fungicide or two applications of mixtures between AP and other fungicides per season were strictly enforced, resistance levels could stay comparatively low despite up to nine accumulative applications of AP to control *B. cinerea*, indicating that the resistance problem of AP might be avoided by using them properly (Gauthier et al. 2003; Hilber and Hilber-Bodmer 1998). Boscalid is a new site-specific fungicide, and it was just registered in China around 2008 to control *B. cinerea* (Wang et al. 2009; Zhang et al. 2007). It was interesting to detect two Bos^R isolates in this study, which confirmed the emergence of resistance to boscalid in Hubei only 4 years after its introduction. To the best of our knowledge, this is the first report of boscalid resistance discovered in *B. cinerea* in China. Studies of boscalid-resistance risk were also conducted in several other countries. For example, isolates resistant to boscalid were detected only 2 years after the application of SDHI in Greece, France, and Germany (Leroch et al. 2011; Veloukas et al. 2011; Walker et al. 2013). All these studies suggested that *B. cinerea* was a high-risk pathogen for the development of boscalid resistance.

A high frequency of isolates with multiple resistances to carbendazim and cyprodinil was observed among the locations tested. A similar finding was also reported in the isolates collected from tomato on Crete Island, where the most common phenotype was the dual resistance to carbendazim and cyprodinil (48.8%) (Konstantinou et al. 2015). In contrast, in spite of using the mixture of carbendazim and diethofencarb to control gray mold in China since 2000 (Zhang et al. 2009b), a comparatively low frequency of resistance was detected in this study. Previous studies have indicated that resistance to carbendazim is associated with mutations in the *TUB2* gene (Malandrakis et al. 2011; Yarden and Katan 1993). In our study, E198V was the predominant mutation in *TUB2* because it was found in 10 of 13 resistant isolates, whereas the E198A mutation only existed in the other 3 isolates. These results were consistent with another study, which reported that all *B. cinerea* isolates resistant to carbendazim carried the E198V mutation (Cai et al. 2015). It would be interesting to further demonstrate why E198V is prevalent compared with E198A in the future. Analysis of the sequence of *TUB2* in the five Car^RDie^R isolates revealed that the E198K mutation occurred in all five isolates. A similar observation was also reported by Liu et al. (2016), who discovered that Car^RDie^R isolates carried the E198K mutation. Resistance to boscalid is conferred by several single amino acid changes in the SdhB or SdhD subunit in *B. cinerea* (Lalève et al. 2014b; Leroux et al. 2010). The two Bos^R isolates found in this study both showed the H272R substitution. Alterations at codon 272 in *B. cinerea* (e.g., H272Y and H272R) were the most frequent genotype in Bos^R isolates. Between the two genotypes, H272R was the predominant one in strawberry fields in the United States and Greece (Fernández-Ortuño et al. 2012; Veloukas et al. 2011).

Many studies demonstrated that the risk of resistance development in *B. cinerea* to fludioxonil is low (Baroffio et al. 2003; Fernández-Ortuño et al. 2013b). This also was confirmed by our results because no resistant isolates were detected among all the tested isolates. However, fludioxonil-resistant isolates were detected from fields in France, Germany, and the United States recently (Fernández-Ortuño et al. 2013a,b, 2014b,c; Kretschmer et al. 2009). Therefore, attention still needs to be paid whenever fludioxonil is applied.

Fitness parameters are essential factors affecting the build-up of a fungicide-resistant population in a fungal pathogen (Brent et al. 1998). After 10 transfers on fungicide-free PDA, the isolates still retained the resistance, indicating that the resistances observed in this study were stable. Assessment of fitness parameters showed no significant differences between the sensitive group, the Car^R group, and the Car^RDie^RCyp^R group in terms of mycelial growth, osmotic sensitivity to NaCl, sporulation both in vitro and in vivo, and virulence, which suggested that resistant isolates from the two resistant groups had fitness similar to that of the sensitive isolates. This result was consistent with previous studies (Malandrakis et al. 2011; Sun et al. 2010). Comparing the fitness parameters between the Bos^R group and Bos^S group revealed that there were no significant differences observed in all the tested parameters except virulence, in which the resistant group showed decreased virulence compared with the sensitive group. Such a result is different from that of a previous study conducted by Kim and Xiao (2011), which reported that the pathogenicity and virulence of Bos^R isolates were not significantly different from the sensitive isolates on apple fruit. The variation between these two results may result from the different substitution at codon 272 in the resistant isolates. In this study, both of the resistant isolates contained the H272R mutation. Previous studies demonstrated that the H272Y mutant showed little fitness penalty. However, the H272R mutant had SDH activity and respiration levels that were only half those in the wild type, and suffered a great fitness penalty (Lalève et al. 2014a).

Regarding the control efficacy evaluation, all the tested fungicides showed high efficacy to control the sensitive isolates while they failed to control the resistant isolates, even for the new fungicide boscalid. Such failure of boscalid to control Bos^R isolates of gray mold in vivo was also found in another report, which declared that control efficacy for the resistant isolates was 6.6 to 11.0% (Vitale et al. 2016). This result gave a strong warning to reduce or even stop using these fungicides at locations where the resistance was detected in Hubei Province.

Continuous monitoring of the development of fungicide resistance is useful in managing *B. cinerea*. Moreover, ignoring the existing problems may lead to the spread of fungicide-resistant populations, thereby increasing control cost, increasing production losses, decreasing crop quality, and polluting the environment. Field resistance of *B. cinerea* to carbendazim, diethofencarb, and cyprodinil and even to the new fungicide boscalid has occurred in the monitored greenhouses in Hubei. This finding challenges the current management of *B. cinerea*, which largely depends on application of these fungicides. In order to control *B. cinerea* more effectively, new antiresistance strategies should be developed. The fungicide spray should be restricted, and alternate fungicides with different mode of action such as fludioxonil ought to be recommended. In addition, biological control agents should be introduced. For example, *Trichoderma harzianum* is one of the ideal candidates because it is effective to control *B. cinerea* (De Meyer et al. 1998). In addition, agricultural measures (e.g., cultivation and phytosanitation) which could decrease the relative humidity of greenhouses and the disease inoculum should be adopted in the integrated management strategy.

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