Sensitivity of *Botrytis cinerea* From Nectarine/Cherry in China to Six Fungicides and Characterization of Resistant Isolates

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Abstract

Botrytis cinerea, the causal agent of gray mold, can result in considerable preharvest and postharvest losses in many economically valuable plant species. Fungicides were widely used to minimize such losses, but fungicide resistances were detected frequently. In the present study, we collected 164 isolates from nectarine and cherry in China and tested the sensitivity to six fungicides. Among the tested isolates, 71 (43.3%) were resistant to azoxystrobin, 14 (8.5%) to cyprodinil, 7 (4.3%) to boscalid, 4 (2.4%) to carbendazim, 1 (0.6%) to iprodione, and no isolates were found to be resistant to fludioxonil. The EC₅₀ value and resistance factor (RF) of resistant isolates were determined. Fitness analysis showed that there were no significant differences between sensitive and resistant isolates

for osmotic stress and pathogenicity, while more conidia production was observed for some resistant isolates. Control efficacy of fungicides showed that the resistant isolates could not be controlled efficiently by using corresponding fungicides. The point mutation G143A was detected in the *Cyt b* gene of the isolates resistant to azoxystrobin, while the point mutation H272R of *SdhB* gene was confirmed in boscalid-resistant isolates, and mutations E198V/A of *TUB2* gene and mutation I365S of *BcOs1* occurred in carbendazim-resistant and iprodione-resistant isolates, respectively. These results indicate that the occurrence of fungicide resistance greatly threatens the management of gray mold on stone fruits nectarine and cherry.

Gray mold, caused by *Botrytis cinerea* Pers.:Fr (teleomorph *Botryotinia fuckeliana* (de Barry) Whetzel), is one of the most destructive plant diseases, often causing huge losses in many economically valuable plant species including vegetables, fruits, and ornamental plants, either in the field or after harvest (Leroux 2007; Williamson et al. 2007). *B. cinerea* has a variety of modes of attack, diverse hosts, and can survive as mycelia, conidia, or sclerotia that can prolong the survival time. In China, stone fruits such as peach, nectarine, and cherry are frequently damaged by *B. cinerea*, which causes pre- and postharvest losses (Shang 2016). In 2002, a survey made in Shanxi province revealed that gray mold caused by *B. cinerea* was the second most serious disease on greenhouse nectarine (Jiao 2004). A survey made during 1997–1999 in Zhejiang province indicated that the average disease incidence of peach gray mold could be 30.5–51%, even 70% in some orchards (Wang and Wang 2001). The control of gray mold is based on integrated management strategies such as removal of diseased tissues, flower and fruit thinning, suitable irrigation and fertilizer application, avoiding intercropping of *B. cinerea*-susceptible plants, etc. (Jiao 2004). Nevertheless, the application of chemical fungicides remains the main approach to control *B. cinerea* worldwide (Fan et al. 2017; Fernández-Ortuño et al. 2015; Hahn 2014; Leroux 2007). The fungicides used to control gray mold belong to several groups, such as methyl benzimidazole carbamates (MBCs), dicarboximide (DCFs), anilino-pyrimidines (APs), quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs), and phenylpyrroles (PPs).

Many of the fungicides are considered to be environmentally friendly due to their higher efficacy, lower toxicity, and lower application rates; however, these advantages are somewhat offset by the vulnerability of *B. cinerea* to resistance development. Because of the wide host range, short life cycle, prolific reproduction, and high genetic variability, *B. cinerea* is considered to be a high-risk fungal pathogen in the development of fungicide resistance (Fernández-Ortuño et al. 2015; Hahn 2014; Leroux et al. 2002). The most common disease management practice consists of the alternation or tank mixtures of fungicides with different modes of action.

Multifungicide resistance was commonly found in *B. cinerea* field isolates from different countries soon after fungicides were used to control gray mold (Baroffio et al. 2003; Chen et al. 2016; Fan et al. 2016; Leroux et al. 2002), and the resistance of *B. cinerea* often compromises the control efficacy and results in the failure of disease control.

MBCs target the fungal cytoskeleton by interfering with assembly of β -tubulin subunit (Albertini et al. 1999; Leroux et al. 2002; Ma et al. 2003). Generally, the mutations at codons 198 (E198A/V/K/Q) and 200 (F200Y) in the β -tubulin gene (*TUB2*) were associated with the resistance in field isolates of many plant pathogens such as *Penicillium expansum* (Cabañas et al. 2009), *Monilinia fructicola* (Chen et al. 2013; Ma et al. 2003), *Tapesia yallundae*, *Tapesia aculeiformis* (Albertini et al. 1999), *Venturia inaequalis* (Koenraadt et al. 1992), and *Neurospora crassa* (Fujimura et al. 1992). In *B. cinerea*, the resistance was generally conferred by mutations of E198A, E198V, or E198K (Fan et al. 2016; Leroux et al. 2002). In addition, other mutations of *TUB2* genes were also reported for conferring resistance to MBCs (Yang et al. 2015). The *N*-phenylcarbamate fungicide diethofencarb is inactive against benzimidazole-sensitive isolates, but exhibits fungicidal activity against resistant ones with mutations E198A/V of *TUB2* gene (Fan et al. 2016; Fujimura 1994). The field isolates with dual resistance to MBCs and diethofencarb have been found harboring F200Y or E198K mutation (Albertini et al. 1999; Banno et al. 2008; Leroux et al. 2002).

DCFs, including iprodione, have been used to control *B. cinerea* since the 1970s, and the resistance has emerged in different areas (Banno et al. 2008; Hahn 2014). Although the mode of action remains unclear, genetic analysis of DCF-resistant *B. cinerea* isolates revealed that the resistance was frequently related to the *BcOs1* gene, which encodes the osmosensing histidine kinase (also called *Daf1*) (Faretra and Pollastro 1991; Oshima et al. 2002). *B. cinerea* isolates

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with different resistance levels to DCFs have been found to contain one or several mutations in *BcOs1*. The most frequent mutation was located at position 365 (I365S/N/R) conferring low resistance (Banno et al. 2008; Grabke et al. 2014). The mutations Q369H/P or N373S exhibited intermediate or high resistance levels (Grabke et al. 2014; Oshima et al. 2006).

Cyprodinil is one of the AP fungicides. It has been suggested that APs inhibit the biosynthesis of methionine and other amino acids (Masner et al. 1994). Resistance of *B. cinerea* to APs has been reported in many studies (Bardas et al. 2008; Weber 2011; Zhang et al. 2009), indicating the high risk for the development of resistance. A recent study showed that the APs resistance in *B. cinerea* is linked to mitochondrial function (Mosbach et al. 2017).

Molecular mechanisms associated with QoI resistance have been reported as point mutations in the mitochondrial cytochrome b (*Cyt b*) gene (Fernández-Ortuño et al. 2008; Grasso et al. 2006; Ishii et al. 2009; Luo et al. 2010). In general, field resistance to QoIs in *B. cinerea* was caused by the mutations G143A, F129L, and G137R in the *Cyt b* gene (Leroch et al. 2013). Isolates with G143A exhibit high levels of resistance that were always associated with the failure of QoIs, whereas isolates with the mutations F129L or G137R exhibit moderate resistance (Gisi et al. 2002; Sierotzki et al. 2007).

The target of the SDHs such as boscalid is the succinate dehydrogenase (SDH) complex, which is a functional part of the mitochondrial electron transport chain. The SDH complex contains four subunits: SdhA, SdhB, SdhC, and SdhD (Hagerhall 1997). Resistance to SDHs is mediated by point mutations which lead to amino acid substitutions at SdhB, SdhC, and SdhD subunits (Sierotzki and Scalliet 2013; Veloukas et al. 2013). Previous studies have shown that mutations H272Y/R/L, P225L/F/T, and N230I in *SdhB* gene were correlated with boscalid resistance (Fernández-Ortuño et al. 2012, 2017; Leroux et al. 2010; Veloukas et al. 2011, 2013; Yin et al. 2011). An additional mutation, H132R, was detected in *SdhD* gene (Leroux et al. 2010).

The PP fungicide fludioxonil is highly effective in inhibiting spore germination and mycelial growth of *B. cinerea* (Leroux et al. 2002), and has broad-spectrum activity against fungal plant pathogens. The fludioxonil-resistant isolates were rarely detected in the field (Fan et al. 2016; Fernández-Ortuño et al. 2013b; Yin et al. 2015). Although some *B. cinerea* isolates were found to be resistant to fludioxonil, no highly resistant isolates were described (Fernández-Ortuño et al. 2013a; Leroch et al. 2013; Li et al. 2014; Vignutelli et al. 2002; Zhao et al. 2010). The mode of action is still unclear for fludioxonil, but several studies showed that fludioxonil resistance is related to the Hog1-type mitogen-activated protein kinase (MAPK) (Kojima et al. 2004; Yoshimi et al. 2005; Zhang et al. 2002). Other studies showed that there were at least two different genes to be linked with the fludioxonil resistance (Vignutelli et al. 2002), and some genes involving salt tolerance also contributed to the resistance to fludioxonil (Hagiwara et al. 2009; Segmüller et al. 2007; Yan et al. 2011; Yang et al. 2012).

Considering that fungicides are widely used to control fruit diseases including gray mold in China, it is very important to assess the fungicide sensitivity in *B. cinerea* populations and trace the resistance shifts. The objectives of this study were to determine the sensitivities of *B. cinerea* isolates from nectarine and cherry in China to the MBC carbendazim, the DCF iprodione, the AP cyprodinil, the QoI azoxystrobin, the SDHI boscalid, and the PP fludioxonil, and to characterize resistant isolates, so that proper strategies could be established for gray mold control.

Materials and Methods

Fungal isolates. A total of 164 *B. cinerea* isolates were isolated from preharvested nectarine at one location in Hubei (13), or preharvested cherry at five locations in Sichuan (60), seven locations in Chongqing (85), and two locations in Yunnan (6) provinces of China in 2014. Briefly, the cotton swab touched the parts of fruits to catch conidia, then touched the surface of potato dextrose agar medium (PDA) containing streptomycin sulfate (100 µg/ml), and single-

spore isolates were obtained according to the method described previously (Luo et al. 2002). Only one isolate was obtained from each fruit. All the 164 isolates were identified to be *B. cinerea* by their morphological characteristics and molecular methods developed previously (Fan et al. 2015).

Fungicide sensitivity tests in vitro. The technical grade fungicides carbendazim (active ingredient [a.i.], 98.1%, Tianjin Jinbei Fine Chemicals CO., LTD), diethofencarb (a.i., 95.4%, Jiangsu Lanfeng Biochemical CO., LTD), iprodione (a.i., 95.3%, Jiangxi Heyi Chemicals CO., LTD), and fludioxonil (a.i., 96.0%, Hubei Jianyuan CO., LTD), cyprodinil and boscalid (a.i., 98.1% and 97%, respectively, Hubei Kangbaotai Fine Chemicals CO., LTD), and azoxystrobin (a.i., 96.0%, Jiangsu Frey Agrochemical CO., LTD) were used in this study. The sensitivity of *B. cinerea* isolates to fungicides was assayed by investigating the inhibition of mycelial growth. Isolates were determined as resistant or sensitive by using the discriminatory concentration (a concentration that fully inhibits mycelial growth of the sensitive isolates). The discriminatory concentrations were 1, 5, 4, 10, 10, and 0.1 µg/ml for carbendazim, iprodione, cyprodinil, azoxystrobin, boscalid, and fludioxonil, respectively, which were determined based on preliminary experiments as well as the discriminatory doses described previously (Amiri et al. 2013; Fan et al. 2016; Fernández-Ortuño et al. 2014). Fungicides were dissolved in 100% acetone, except for carbendazim, which was dissolved in lactic acid, and adjusted to appropriate concentration for stock. The concentration of stock solution was 10,000 µg/ml for iprodione, 4,000 µg/ml for azoxystrobin, 200 µg/ml for fludioxonil, and 2,000 µg/ml for other used fungicides. The salicylhydroxamic acid (SHAM) was dissolved in acetone to make 40,000 µg/ml stock solution. The stock solution was added to sterilized media to produce a final concentration. The fungicide cyprodinil was added to CzA medium (0.2% sodium nitrate, 0.05% potassium chloride, 0.05% magnesium sulfate, 0.001% ferric sulfate, 0.1% dipotassium phosphate, 0.001% zinc sulfate, 0.0005% copper sulfate, 3% sucrose, and 1.5% agar), boscalid to YBA (1% yeast extract, 1% Bacto peptone, 2% sodium acetate, 1.5% agar), and fludioxonil to MM medium (1% glucose, 0.15% dipotassium phosphate, 2% monopotassium phosphate, 0.1% ammonium sulfate, 0.2% yeast extract, 0.05% magnesium sulfate, 1.25% agar); others were added to PDA medium. For azoxystrobin, the plates were also amended with 80 µg/ml SHAM to inhibit the alternative respiratory pathway (Bardas et al. 2010). Isolates that grew on the fungicide-amended media were designated as resistant, while those unable to grow were designated as sensitive. The experiment was conducted three times.

The effective concentration of fungicides that inhibits mycelial growth by 50% (EC₅₀) was determined for 10 sensitive and several resistant isolates. The fungicide concentrations were shown in Supplementary Table S1. The plates with or without fungicides were inoculated with a 5-mm-diameter mycelial plug cut from the edge of actively growing culture of each isolate. Then plates were incubated in the dark at 22°C for 5 days. The colony diameter was measured in two perpendicular directions. The EC₅₀ values were assessed by regressing percentage of mycelial growth inhibition against the log₁₀ of fungicide concentrations. The resistance factor was expressed as the EC₅₀ value of resistant isolate divided by the mean EC₅₀ value of corresponding sensitive isolates.

Evaluation of fitness of resistant isolates. The resistant isolates to individual fungicides were selected for fitness evaluation. Generally, three isolates were used for each group of resistant isolates. For carbendazim or cyprodinil resistant isolates, only two isolates were used because the multiple resistant isolate BSC14-31a was also resistant to carbendazim and cyprodinil. For investigating mycelial growth, 5 mm mycelial plugs were transferred to the fresh PDA plates and incubated in the dark at 22°C for 2 days. The colony diameter was measured in two perpendicular directions for each treatment. Sensitivity to osmotic stress was determined by measuring inhibition of mycelia. The mycelial plugs were transferred to PDA amended with 1 M KCl or glycerol. The plates were incubated in the dark at 22°C for 2 days, and the colony diameter was measured as described

above. The relative inhibition of mycelial growth was calculated by the formula: one minus the mean diameter of treatment divided by the control. For each isolate, four replicate plates were used.

In order to determine pathogenicity, nectarines of similar size and maturity were selected and washed three times with tap water. Then the nectarines were disinfected by immersing in 5% (vol/vol) sodium hypochlorite solution for 15 min and rinsed three times in sterile deionized water, and subsequently air-dried. The nectarines were wounded by a cork borer, and the same diameter mycelial plugs were inoculated. The nectarines were placed in plastic boxes with wet absorbent paper at the bottom. The boxes were covered by cling film to maintain high relative humidity and incubated at 22°C for 3–4 days. The pathogenicity was assessed by measuring the diameter of the lesions. The cling film was removed and fruit were further incubated for 2 weeks to produce spores, which were harvested by washing inoculated nectarines, and conidia concentration was determined with a hemacytometer.

For evaluation of the control efficacy of resistant isolates, each nectarine was sterilized as describe above and sprayed with 10 ml of the fungicide suspensions that were diluted 1,000–2,000 times of corresponding commercial fungicides according to the manufacturer's instructions. Subsequently, fruit were air-dried and inoculated as previously. Three replicates were used in each treatment, and the experiment was repeated three times independently. The lesion diameter was measured after 3 days. Inhibition rate of mycelial growth = 1 – the mean diameter of treatment with fungicides divided by the mean diameter of control without fungicides.

Molecular analysis of the fungicide target genes in resistant isolates. The isolates were transferred to PDA medium, on which the cellophane membrane was set, and incubated for 3 days. The mycelia were harvested, and genomic DNA was extracted using the Easypure Plant Genomic DNA Extraction Kit (TransGen Biotech). The partial target genes were amplified with the corresponding primers (Supplementary Table S2). PCR volumes were 50 µl, containing 1× PCR buffer, 10–50 ng DNA, 0.4 µM each primer, 100 µM each dNTP, and 1 U of Taq DNA polymerase (TAKARA, Biotech Co.). All amplifications were performed in an iCycler thermal cycler (Bio-Rad Laboratories Inc.). The PCR reaction was performed with the following parameters: an initial preheating for 4 min at 94°C, followed by 30 cycles of denaturation at 94°C for

30 s, annealing at 50–60°C for 30 s, and extension at 72°C for 1 min or more times according to the sequence length; with a final extension at 72°C for 10 min. PCR products were sequenced to detect whether mutations exist in corresponding target genes after checked by electrophoresis.

Results

Resistance profiles of *B. cinerea* isolates to six fungicides. Altogether, 164 isolates of *B. cinerea* were obtained and evaluated for sensitivity to six fungicides, including carbendazim, iprodione, cyprodinil, azoxystrobin, boscalid, and fludioxonil. Among the tested isolates, 4 (2.4%) were resistant to carbendazim, 1 (0.6%) to iprodione, 14 (8.5%) to cyprodinil, 71 (43.3%) to azoxystrobin, 7 (4.3%) to boscalid, and no isolates were found to be resistant to fludioxonil (Table 1). Among the 164 isolates of *B. cinerea* obtained in the study, 10 sensitive isolates and several resistant isolates were selected to determine the EC₅₀ values for carbendazim, iprodione, cyprodinil, azoxystrobin, and boscalid by using mycelial growth inhibition assays. Based on EC₅₀ values, the resistance factor (RF) of resistant isolates was calculated. As a result, high RFs were observed in selected resistant isolates, especially in the multiple resistant isolate BSC14-31a, which showed the highest RF value (Table 2).

Fitness of the resistant isolates. Three sensitive isolates (BN2, BSC14-26b, and BCQ14-221) and 11 resistant isolates (Car^R isolates BSC14-108a and BSC14-24a, Cyp^R isolates BSC14-45a and BSC14-95a, Azo^R isolates BCQ14-223a, RC7-1, and QJ12-1, Bos^R isolates BSC14-94b, QJ3-2, and QJ6-1, and Azo^RCyp^RCar^RDie^RIpr^R isolate BSC14-31a) were selected to evaluate whether there exist fitness penalties in resistant isolates. Results showed that no significant differences for mycelial growth and osmotic sensitivity were observed between sensitive and resistant isolates, although some significant differences were observed between individual isolates. Four of 11 resistant isolates showed the tendency to produce more spores on nectarine fruit, especially isolates BSC14-31a and BSC14-24a, indicating the different sporulation in individual isolates. The resistant isolates did not show significant difference with sensitive isolates for pathogenicity (Table 3).

Control efficacy of fungicides to resistant isolates. The control efficacy of iprodione, cyprodinil, azoxystrobin, and boscalid were evaluated for resistant isolates, and sensitive isolates were used as

Table 1. The frequency and distribution for resistant isolates to different fungicides

Fungicides	Isolates (resistant isolates) ^z				Number of resistant isolates	Number of isolates	Resistance frequency
	Sichuan	Chongqing	Yunnan	Hubei			
Carbendazim	60 (3)	85 (1)	6 (0)	13 (0)	4	164	2.4%
Iprodione	60 (1)	85 (0)	6 (0)	13 (0)	1	164	0.6%
Cyprodinil	60 (8)	85 (6)	6 (0)	13 (0)	14	164	8.5%
Azoxystrobin	60 (24)	85 (42)	6 (2)	13 (3)	71	164	43.3%
Boscalid	60 (1)	85 (6)	6 (0)	13 (0)	7	164	4.3%
Fludioxonil	60 (0)	85 (0)	6 (0)	13 (0)	0	164	0

^z The number in parentheses means resistant isolates.

Table 2. The EC₅₀ and RF values of sensitive and resistant isolates

Isolates	Phenotype ^y	EC ₅₀ (µg/ml)					RF ^x				
		Azo	Bos	Car	Cyp	Ipr	Azo	Bos	Car	Cyp	Ipr
S ^z	Azo ^S Bos ^S Cyp ^S Car ^S Ipr ^S	1.37	3.26	0.07	0.02	0.47	-	-	-	-	-
BCQ14-152a	Car ^R Die ^R	-	-	7.72	-	-	-	-	108.85	-	-
BSC14-108a	Car ^R Die ^S	-	-	17.39	-	-	-	-	245.14	-	-
BCQ14-223a	Azo ^R	6	-	-	-	-	4.37	-	-	-	-
RC8-1	Bos ^R	-	24.63	-	-	-	-	7.55	-	-	-
BSC14-94b	Bos ^R	-	47.06	-	-	-	-	14.44	-	-	-
BSC14-31a	Azo ^R Cyp ^R Car ^R Die ^R Ipr ^R	88.46	-	22.22	6.58	3.45	64.47	-	313.35	299.65	7.28

^x RF (resistance factor) = the EC₅₀ value of resistant isolate divided by the mean EC₅₀ values of sensitive isolates.

^y Azo = azoxystrobin, Bos = boscalid, Cyp = cyprodinil, Car = carbendazim, Die = diethofencarb, Ipr = iprodione; R = resistant, S = sensitive.

^z S Means 10 sensitive isolates that were used for calculating EC₅₀ values.

a control. Results showed that the sensitive isolates could be controlled successfully by fungicides except for azoxystrobin, which exhibited a lower efficacy compared with the other three fungicides. However, the control efficacies were very low for resistant isolates, indicating that fungicides could not control the corresponding resistant isolates efficiently. As shown in Table 4, the multiple resistant isolate BSC14-31a could not be controlled by any of the three fungicides iprodione, azoxystrobin, and boscalid. For the cyprodinil and boscalid treatments, the control efficacy showed significant difference between corresponding resistant and sensitive isolates. For azoxystrobin treatment, low resistant isolates BSC14-223a, RC7-1, and QJ12-1 showed similar growth rate with sensitive isolates, but highly resistant isolate BSC14-31a showed a better growth compared with no fungicide treatment, indicating a negative control efficacy (Table 4).

Diethofencarb generally showed negative cross resistance with MBC fungicides. Therefore, the mixture of diethofencarb and carbendazim was used for evaluation of control efficacy. In this study, the carbendazim-resistant isolates BSC14-108a and BSC14-24a, and carbendazim and diethofencarb dual resistant isolate BSC14-31a, were evaluated with three sensitive isolates, BN2, BSC14-26b, and BCQ14-221, as controls. When three sensitive isolates were controlled by carbendazim or a mixture of carbendazim and diethofencarb successfully, BSC14-108a and BSC14-24a could only be controlled by a mixture of carbendazim and diethofencarb with control efficacy of 60.69% and 65.53%, but not by solo carbendazim (control efficacy –22.94% and –13.65%). Neither carbendazim nor a mixture of carbendazim and diethofencarb could control BSC14-31a successfully, as reflected by the low efficacy of –4.33% and 36.49%, respectively (Table 5).

Table 3. Mycelial growth, osmotic sensitivity, sporulation, and virulence of resistant isolates

Isolates	Phenotype ^x	PDA			Nectarine	
		Colony diameter (cm)	Inhibition rate ^y (%)		Lesion diameter (cm)	Sporulation (10 ⁵ conidia/ml)
			KCl	Glycerol		
BN2	S	6.56 ± 1.05 ab ^z	53.58 ± 0.93 abc	41.58 ± 2.34 ab	3.63 ± 0.64 a	0.23 ± 0.06 d
BSC14-26b	S	5.94 ± 0.68 abcd	47.52 ± 8.63 abcd	31.32 ± 7.86 bcd	3.58 ± 0.59 a	0.16 ± 0.22 d
BCQ14-221	S	5.53 ± 0.31 bcd	50.69 ± 3.01 abcd	23.55 ± 4.75 d	3.02 ± 0.45 a	0.12 ± 0.6 d
		6.01 ± 0.15 A	50.59 ± 2.76 AB	32.15 ± 4.14 B	3.41 ± 0.16 A	0.36 ± 0.39 CD
BSC14-108a	Car ^R Die ^S	6.62 ± 0.91 a	48.75 ± 0.92 abcd	30.50 ± 5.14 bcd	3.17 ± 0.29 a	0.56 ± 0.24 cd
BSC14-24a	Car ^R Die ^S	5.55 ± 2.15 abcd	45.98 ± 1.99 abcd	35.83 ± 1.62 abc	3.33 ± 0.32 a	2.89 ± 1.02 b
		5.98 ± 0.18 A	47.84 ± 3.38 BC	31.16 ± 5.07 BC	3.22 ± 0.19 A	1.91 ± 0.48 ABC
BSC14-45a	Cyp ^R	5.54 ± 1.36 abcd	35.13 ± 3.42 d	27.02 ± 2.17 cd	3.84 ± 0.44 a	0.17 ± 0.05 d
BSC14-95a	Cyp ^R	5.25 ± 0.21 cd	41.62 ± 3.66 bcd	46.19 ± 3.12 a	3.42 ± 0.56 a	0.69 ± 0.11 cd
		5.29 ± 0.18 BC	38.85 ± 3.38 C	34.60 ± 5.07 A	3.60 ± 0.19 A	0.28 ± 0.48 D
BCQ14-223a	Azo ^R	6.30 ± 1.40 abc	61.36 ± 1.90 a	28.72 ± 2.70 cd	3.33 ± 0.15 a	1.58 ± 1.37 bcd
RC7-1	Azo ^R	5.43 ± 0.99 cd	51.76 ± 3.11 abc	28.63 ± 3.61 cd	3.75 ± 0.55 a	0.27 ± 0.14 d
QJ12-1	Azo ^R	5.69 ± 1.66 abcd	60.6 ± 4.11 a	22.88 ± 2.13 d	3.69 ± 0.18 a	0.62 ± 0.22 cd
		5.80 ± 0.15 AB	57.90 ± 2.76 A	26.74 ± 4.14 D	3.59 ± 0.16 A	0.70 ± 0.39 BCD
BSC14-94b	Bos ^R	5.78 ± 1.19 abcd	56.18 ± 7.77 ab	32.03 ± 1.83 bcd	3.40 ± 0.61 a	2.44 ± 0.75 b
QJ3-2	Bos ^R	5.31 ± 1.76 cd	41.00 ± 3.65 bcd	28.29 ± 3.33 cd	3.64 ± 0.76 a	1.86 ± 0.64 bc
QJ6-1	Bos ^R	5.53 ± 0.98 bcd	48.77 ± 2.43 abcd	30.87 ± 1.75 bcd	3.72 ± 0.79 a	0.7 ± 0.12 cd
		5.52 ± 0.15 AB	48.65 ± 2.76 BC	30.39 ± 4.14 C	3.58 ± 0.16 A	1.56 ± 0.39 ABC
BSC14-31a	Azo ^R Car ^R Die ^R Cyp ^R Ipr ^R	5.15 ± 0.12 d	39.44 ± 6.86 cd	24.76 ± 6.85 cd	3.36 ± 0.64 a	4.67 ± 1.78 a

^x Car = carbendazim, Die = diethofencarb, Cyp = cyprodinil, Azo = azoxystrobin, Bos = boscalid, Ipr = iprodione; S = sensitive, R = resistant.

^y Inhibition rate of mycelial growth = 1 – the mean diameter of treatment with KCl or glycerol divided by the control.

^z Data shown are the means of two independent experiments. Mean ± standard deviation; bold values are means of individual phenotype groups, values followed by the same letter within a column for each experiment separately are not significantly different according to the one-way analysis of variance with least significant difference test at *P* = 0.05.

Table 4. Control efficacy of azoxystrobin, boscalid, cyprodinil, and iprodione to sensitive and resistant strains

Isolates	Phenotype ^x	Lesion diameter (cm)					Inhibition ^y (%)			
		CK	Azo	Bos	Cyp	Ipr	Azo	Bos	Cyp	Ipr
BN2	S	3.03 ± 0.64 ^z	1.28 ± 0.15	0.64 ± 0.60	0.34 ± 0.24	0.41 ± 0.08	57.69 ± 4.79 b	79.58 ± 19.94 a	88.64 ± 8.04 b	86.45 ± 2.48 a
BSC14-26b	S	2.98 ± 0.59	1.62 ± 0.09	0.97 ± 0.36	0.12 ± 0.17	0.87 ± 0.23	45.81 ± 3.11 d	67.60 ± 12.09 c	95.90 ± 5.71 a	70.76 ± 7.83 a
BCQ14-221	S	2.42 ± 0.45	1.22 ± 0.22	0.54 ± 0.26	0.31 ± 0.36	0.63 ± 0.38	49.54 ± 8.95 c	77.52 ± 10.53 b	87.16 ± 14.70 c	74.08 ± 15.73 a
							51.013 ± 4.24 A	74.90 ± 5.64 A	90.56 ± 2.79 A	77.09 ± 4.21 A
BSC14-45a	Cyp ^R	2.94 ± 0.42	-	-	1.29 ± 0.81	-	-	-	56.13 ± 4.19 e	-
BSC14-95a	Cyp ^R	2.82 ± 0.71	-	-	0.95 ± 0.51	-	-	-	66.32 ± 14.13 d	-
									61.22 ± 3.94 B	
BCQ14-223a	Azo ^R	2.73 ± 0.15	1.07 ± 0.04	-	-	-	60.90 ± 1.62 a	-	-	-
RC7-1	Azo ^R	2.89 ± 0.73	1.21 ± 0.14	-	-	-	58.14 ± 2.34 b	-	-	-
QJ12-1	Azo ^R	2.79 ± 0.52	1.52 ± 0.34	-	-	-	45.52 ± 4.79 d	-	-	-
							54.853 ± 3.01 A			
BSC14-94b	Bos ^R	2.80 ± 0.61	-	2.01 ± 0.81	-	-	-	25.20 ± 28.77 f	-	-
QJ3-2	Bos ^R	3.06 ± 0.74	-	2.06 ± 0.94	-	-	-	32.68 ± 3.44 d	-	-
QJ6-1	Bos ^R	3.19 ± 0.23	-	2.19 ± 0.31	-	-	-	31.35 ± 19.55 e	-	-
								29.74 ± 3.98 B		
BSC14-31a	Azo ^{HR} Cyp ^R Ipr ^R	2.76 ± 0.64	3.27 ± 0.52	-	2.73 ± 0.34	1.96 ± 0.27	-18.75 ± 18.70 e	-	1.01 ± 12.46 f	28.83 ± 9.68 b

^x Cyp = cyprodinil, Azo = azoxystrobin, Bos = boscalid, Ipr = iprodione; S = sensitive, R = resistant, HR = highly resistant.

^y Inhibition rate of mycelial growth = 1 – the mean diameter of treatment with fungicides divided by the mean diameter of control without fungicides.

^z Data shown are the means of two independent experiments. Mean ± standard deviation; bold values are means of individual phenotype groups, values followed by the same letter within a column for each experiment separately are not significantly different according to the one-way analysis of variance with least significant difference test at *P* = 0.05.

Identification of point mutations in fungicide target genes. To determine whether the resistance was caused by target gene mutations, the target genes for carbendazim, iprodione, azoxystrobin, and boscalid were sequenced in resistant and sensitive isolates. As shown in Table 6, the carbendazim-resistant isolates BSC14-24a, BSC14-31a, and BSC14-152a harbored a mutation at codon 198 of *TUB2* gene with amino acid change from glutamate to valine (E198V). Another carbendazim-resistant isolate, BSC14-108a, that is sensitive to diethofencarb contained a mutation at codon 198 with amino acid change from glutamate to alanine (E198A). In addition, BSC14-31a, that was also resistant to diethofencarb, did not show any other mutations besides E198V. For iprodione resistance, the *BcOs1* gene of resistant isolate BSC14-31a contained a mutation at codon 365 with amino acid change from isoleucine to serine (I365S). For azoxystrobin resistance, the resistant isolate BSC14-31a had a mutation at codon 143 of *Cyt b* gene, resulting in an amino acid change from glycine to alanine (G143A). Mutation was not found in another resistant isolate, BSC14-223a (Table 6). For boscalid resistance, *SdhB* gene of resistant isolate BSC14-94b showed a mutation at codon 272 with amino acid change from histidine to arginine (H272R). Other resistant isolates, QJ6-1, QJ3-2, RC7-1, RC8-1, and QJ8-1, did not show any mutation in the *SdhB* gene.

Discussion

B. cinerea can infect a variety of crops and cause enormous losses. Chemical fungicides have been a powerful tool to control gray mold. Because the fungicides were used extensively, fungicide resistance has emerged and resulted in a serious deficiency of corresponding fungicides.

In this study, 164 *B. cinerea* isolates from nectarine and cherry in four provinces of China were tested for sensitivity to six fungicides. The *N*-phenylcarbamate fungicide diethofencarb generally revealed negative cross resistance with MBC fungicides, and a mixture of carbendazim and diethofencarb was used to manage the MBC fungicide resistance (Leroux et al. 1999; Zhang et al. 2010). The dual resistance to carbendazim and diethofencarb was confirmed in this study, which is consistent with previous reports (Fan et al. 2016; Leroux et al. 2002; Rodríguez et al. 2014).

There were 43.3% isolates resistant to azoxystrobin, which was lower than that observed in Zhejiang province of China from strawberry (Yin et al. 2015). It is reasonable since much of fungicides were applied to strawberry compared with cherry and nectarine. Only a few isolates showed resistance to carbendazim, cyprodinil, and boscalid, although high frequency of such resistance has been reported worldwide (Bardas et al. 2010; Leroch et al. 2013; Rodríguez et al. 2014; Yin et al. 2015), suggesting that these fungicides could still be used to control gray mold in this areas, although the monitoring of the development of resistance should be carried out in a timely manner. The relatively lower resistance frequencies of MBC and other chemical class fungicides compared with QoI fungicides might be caused by the practices that QoI fungicides were frequently recommended to control fungal diseases, e.g., brown rot disease.

Some reports showed the occurrence of fludioxonil resistance in the field (Fernández-Ortuño et al. 2013a; Leroch et al. 2013; Li et al. 2014; Zhao et al. 2010), but we did not detect any isolates showing resistance to it, which is consistent with the previous observations for fludioxonil in *B. cinerea* populations (Fan et al. 2016; Fernández-Ortuño et al. 2013b; Li et al. 2014). Thus, the fludioxonil still could be used in some parts of China to control gray mold on stone fruit. In eastern China, high frequency of multiple resistance to different kinds of fungicides were reported (Yin et al. 2015). We also found an isolate (BSC14-31a) with multiple resistance to carbendazim, diethofencarb, iprodione, cyprodinil, and azoxystrobin, which suggests the high risk for spreading in the stone fruit orchard.

In the current study, the Azo^R isolate BCQ14-223a showed the RF as 4.37, which was much lower than that reported previously in eastern China (Yin et al. 2015; Yin et al. 2012). The reason for lower RF can be explained by the relatively higher EC₅₀ values of sensitive isolates (up to 1.37 µg/ml) which were much higher than those reported previously (De Miccolis Angelini et al. 2012; Yin et al. 2015). The low resistance level of BCQ14-223a was also confirmed in the detached fruit assay with fungicide treatment where the application of azoxystrobin showed high inhibition for this isolate. Similarly, the EC₅₀ values of sensitive isolates to boscalid were higher compared with previous reports (Veloukas et al. 2011; Yin et al. 2011). The probable reason is that different methods were used; they determined

Table 6. The point mutations of target genes for four fungicides

Fungicides	Isolates	Phenotype ^y	Mutations ^z	Target gene
Carbendazim	BCQ14-127a	S	no	<i>TUB2</i>
	BCQ14-175a	S	no	
	BSC14-24a	R	E198V	
	BSC14-31a	R	E198V	
	BSC14-108a	R	E198A	
	BSC14-152a	R	E198V	
Iprodione	QJ1-1	S	no	<i>BcOs1</i>
	BSC14-112a	S	no	
	BSC14-31a	R	I365S	
Azoxystrobin	BHB14-4b	S	no	<i>Cytb</i>
	BCQ14-152a	S	no	
	BCQ14-223a	R	no	
	BCQ14-31a	R	G143A	
Boscalid	RC9-2	S	no	<i>SdhB</i>
	RC7-1	R	no	
	RC8-1	R	no	
	QJ3-2	R	no	
	QJ6-1	R	no	
	QJ8-1	R	no	
	BCQ14-94b	R	H272R	

^y S = sensitive to corresponding fungicides, R = resistant.

^z No means no mutation was detected.

Table 5. Control efficacy of carbendazim and diethofencarb to sensitive and resistant strains

Isolates	Phenotype ^x	Lesion diameter (cm)			Efficiency ^y (%)	
		CK	Car	Car+Die	Car	Car+Die
BN2	Car ^S Die ^R	3.03 ± 0.64 ^z	0.33 ± 0.12	0.77 ± 0.36	89.19 ± 3.90 b	74.54 ± 11.81 c
BSC14-26b	Car ^S Die ^R	2.98 ± 0.59	0 ± 0	0.72 ± 0.48	100 ± 0 a	75.79 ± 16.17 b
BCQ14-221	Car ^S Die ^R	2.42 ± 0.45	0 ± 0	0.53 ± 0.76	100 ± 0 a	78.10 ± 31.26 a
					96.39 ± 2.35 A	73.61 ± 2.20 A
BSC14-108a	Car ^R Die ^S	2.57 ± 0.29	3.21 ± 0.37	0.94 ± 1.15	-22.94 ± 14.46 e	60.69 ± 44.78 e
BSC14-24a	Car ^R Die ^S	2.93 ± 0.32	3.33 ± 0.24	1.01 ± 0.55	-13.65 ± 10.22 d	65.53 ± 18.52 d
					-12.62 ± 2.88 B	61.01 ± 2.69 B
BSC14-31a	Car ^R Die ^R	2.76 ± 0.64	2.91 ± 0.29	1.75 ± 0.33	-4.33 ± 2.35 B	36.49 ± 6.24 C

^x Car = carbendazim, Die = diethofencarb; S = sensitive, R = resistant.

^y Inhibition rate of mycelial growth = 1 - the mean diameter of treatment with fungicides divided by the mean diameter of control without fungicides.

^z Data shown are the means of two independent experiments. Mean ± standard deviation; bold values are means of individual phenotype groups, values followed by the same letter within a column for each experiment separately are not significantly different according to the one-way analysis of variance with least significant difference test at *P* = 0.05.

the inhibition of germ tube growth instead of mycelial growth. According to the RFs, two resistant isolates to boscalid showed low or moderate resistance, although highly resistant isolates have been detected frequently in recent years in other places (Fernández-Ortuño et al. 2012; Veloukas et al. 2011; Yin et al. 2011). This indicates that boscalid is still efficient to control the disease, but there is potential resistance risk.

Our investigation of fitness components including mycelial growth, osmotic sensitivity, and pathogenicity revealed that there was no significant fitness difference between sensitive and resistant isolates. This is consistent with the previous research that plenty of fungicide-resistant *B. cinerea* populations did not show the fitness penalty (Chen et al. 2016; Rupp et al. 2017; Veloukas et al. 2011, 2014), even though some resistant isolates showed reduced fitness values (Bardas et al. 2008; Billard et al. 2012; Lalève et al. 2014; Veloukas et al. 2014). In this study, some resistant isolates showed better sporulation ability compared with sensitive ones, especially for multiple resistant isolate BSC14-31a. It should be noted that generally the DCF resistant isolates show increased sensitivity to osmotic stress. However, the iprodione (DCF) resistant isolate BSC14-31a did not show significant difference with other isolates for osmotic stress; it is possible this isolate could be more tolerant to adverse conditions including osmotic stress. Overall, fitness abilities of the resistant isolates were similar or better than sensitive isolates in the absence of fungicide pressure. This indicates that selection pressure in the field may favor the resistant isolates and make disease management more difficult.

In this study, cyprodinil, iprodione, and boscalid showed better control efficacy than azoxystrobin to the sensitive isolates. Furthermore, the resistant isolate BSC14-31a showed a better growth after azoxystrobin treatment. Considering the high resistance frequency and low inhibiting efficiency to sensitive isolates, azoxystrobin is not suitable for *B. cinerea* control. Iprodione, cyprodinil, and boscalid also lost the ability to control corresponding resistant isolates. Fortunately, the resistance frequencies to these three fungicides were not very high (less than 10%), suggesting that some of these fungicides could still be used, but monitoring of resistance development is needed. At the same time, we detected carbendazim-resistant isolates as well as isolates with dual resistance to carbendazim and diethofencarb. We found that one isolate cannot be controlled by the mixture of carbendazim and diethofencarb, indicating that these tubulin inhibitors should be restricted in the fields. We did not detect fludioxonil-resistant isolates, so application of fludioxonil to control gray mold was highly recommended.

The mutations E198A/V/K/Q and F200Y in the *TUB2* gene are generally associated with MBCs resistance (Banno et al. 2008; Chen et al. 2013; Fan et al. 2016; Leroux et al. 2002; Ma et al. 2003). In this study, the mutations E198A/V were detected in resistant isolates, but no other mutations were found. The diethofencarb can inhibit resistant isolates containing mutations E198A/V of *TUB2*, but is inactive against benzimidazole-sensitive isolates. Interestingly, no other point mutation was found besides E198V in the carbendazim and diethofencarb dual resistance isolate BSC14-31a, which indicated another mechanism was involved.

Although the action mode of DCFs remained unclear, some studies mentioned that the DCFs resistance was associated with mutations in *BcOs1* gene (Faretra and Pollastro 1991; Oshima et al. 2002). The I365S/N/R, Q369H/P, or N373S were described to contribute different levels of resistance (Banno et al. 2008; Grabke et al. 2014; Oshima et al. 2006). In this study, we detected the I365S mutation from the resistant isolate BSC14-31a.

The Azo^R isolate BSC14-31a harbored the point mutation G143A, which has been widely recognized in previous studies (Banno et al. 2009; Yin et al. 2012, 2015). However, we found that another Azo^R isolate, BSC14-223a, did not contain the G143A mutation, indicating that other point mutations or mechanisms are involved in the resistance to QoI fungicides in *B. cinerea*.

For Bos^R isolates, only the highly resistant isolate contained the point mutation H272R in the *SdhB* gene. This is consistent with a previous report that no mutation of *SdhB* was detected in boscalid

moderately resistant isolates (Amiri et al. 2014). Previous studies showed that several point mutations in *SdhB* gene contributed to the boscalid resistance and the *SdhC* and *SdhD* also mediate the resistance (Fernández-Ortuño et al. 2012; Leroux et al. 2010; Veloukas et al. 2011, 2013). The low resistance isolate did not show mutations in *SdhB* gene; other mechanisms might confer such low resistance.

In conclusion, we tested the sensitivity of 164 *B. cinerea* isolates to six fungicides and found that the high frequency of resistance was common. Fitness analysis showed that resistant isolates did not show significant fitness penalty compared with sensitive isolates. In vivo control efficacy evaluation showed that resistant isolates cannot be controlled successfully by the corresponding fungicides. Molecular analysis showed that point mutations in fungicide target genes were the main mechanisms causing the resistance and corresponding control failure. Based on the wide occurrence of fungicide resistance and the small fitness penalty of resistant isolates, the current management strategy should be changed, especially the application of MBCs, DCFs, APs, and QoIs should be restricted, and the PP fungicide fludioxonil should be positively introduced in the management strategies. As with a previous study (Guarnaccia et al. 2014), further studies comparing in vitro with in vivo results and the shift in sensitivity over time should be carried out with a new collection of isolates after new treatments with the same fungicides tested in this study.

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