Combination of Suspension Array and Mycelial Growth Assay for Detecting Multiple-Fungicide Resistance in *Botrytis cinerea* in Hebei Province in China

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Abstract

To provide a high-throughput, efficient, and accurate method to monitor multiple-fungicide resistance of *Botrytis cinerea* in the field, we used the suspension array, sequencing, and mycelial growth assay in our research. Discriminating-dose bioassays for detecting carbendazim, diethofencarb, boscalid, and iprodione resistance (Car^R, Die^R, Bos^R, and Ipr^R, respectively) were used to analyze 257 isolates collected from Hebei Province in China during 2016 and 2017. High resistance frequencies to carbendazim (100%), diethofencarb (92.08%), and iprodione (86.59%) were detected. Bos^R isolates accounted for 11.67% of the total. In addition, 103 isolates were randomly selected for phenotype and genotype detection. The high-throughput suspension array was utilized to detect eight genotypes simultaneously, including *BenA*-E198, *BenA*-198A, *SdhB*-H272, *SdhB*-272Y, *BcOSI*-I365, *BcOSI*-365S, *erg27*-F412, and *erg27*-412S, which were associated with resistance toward carbendazim or diethofencarb, boscalid, iprodione, and fenhexamid (Fen^R), respectively. Most of the benzimidazole-resistant isolates (81.55%) possessed the E198V mutation in the *BenA* gene. Ninety-three isolates with dual

resistance to carbendazim and diethofencarb showed the E198V/K mutation. All Bos^R isolates carried the H272R mutation in the *SdhB* gene. The I365S and Q369P+N373S (66.99%) mutations in the *BcOSI* gene were more frequently observed. No mutation was detected in the *erg27* gene in Hebei isolates. There were 13 resistance profile phenotypes. Phenotypes with triple resistance were the most common (83.50%), and Car^RDie^RBos^RIpr^RFen^S was the major type. Car^R isolates that carried E198V/K/A were all highly resistant (HR) and only one F200Y mutant was moderately resistant (MR) to carbendazim. Isolates that possessed E198V/K were MR or HR to diethofencarb. Bos^R isolates that possessed H272R mutation were lowly resistant (LR). Ipr^R isolates were all LR or MR. The distribution of half maximal effective concentrations of Car^R isolates with E198V/K mutations and Ipr^R isolates with Q369P+N373S mutations significantly increased from 2016 to 2017. Combined with our observations, a combination method of the high-throughput suspension array and the mycelial growth assay was suggested to accurately monitor multiple resistance of *B. cinerea* in the field.

Gray mold caused by the plant pathogen *Botrytis cinerea* is a destructive and ubiquitous disease. The control of gray mold mainly depends on using different chemical fungicides. However, the high rate of reproduction and genetic variation of *B. cinerea*, along with the abuse of fungicides, has resulted in increasing resistance of *B. cinerea* worldwide (De Miccolis Angelini et al. 2014b; Saito et al. 2016).

There are eight major categories of fungicides for controlling *B. cinerea*, including benzimidazoles, *N*-phenylcarbamates, dicarboximides (DCs), anilinopyrimidines (APs), phenylpyrroles, quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs), and sterol biosynthesis inhibitors (SBIs). Both benzimidazoles and *N*-phenylcarbamates inhibit the growth of mycelial and spore germ tubes by binding to the β -tubulin protein in fungi. Therefore, E198A, E198V, E198K, and F200Y mutations in the β -tubulin gene (*BenA*) will greatly reduce the effect of benzimidazoles on *B. cinerea* (Banno et al. 2008; Luck and Gillings 1995; Yarden and Katan 1993). Meanwhile, pathogens with the same mutations of E198V and E198K are resistant to *N*-phenylcarbamates (Beever

et al. 1989; Yourman et al. 2000; Ziogas et al. 2009). Research suggests that dicarboximides interfere with the osmotic signal transduction pathway. The amino acid substitutions in the osmosensing histidine kinase gene (*BcOSI*) were I365S, I365N, I365R, and Q369P+N373S (Cui et al. 2002, 2004; Grabke et al. 2014; Ma et al. 2007; Oshima et al. 2006). APs can be used to control benzimidazoles, *N*-phenylcarbamates, and DC-resistant isolates of *B. cinerea* because there is no cross-resistance with these fungicides (Latorre et al. 2002). Phenylpyrroles constitute a new type of fungicide, and existing theory suggests that they are associated with overactivation of mitogen-activated protein kinases (Bartlett et al. 2002). The fungicidal effects of QoIs and cytochrome b (Cytb) are closely related (Yoshimi et al. 2005). Mutations in key sites of the Cytb gene, such as the G143A mutation, result in fungicide resistance (Ishii et al. 2009; Jiang et al. 2009; Leroy et al. 2013). SDHIs can prevent a variety of diseases, act primarily on the mitochondrial respiratory chain, and interact with the succinate dehydrogenase complex. Resistance to SDHIs occurs after the substitution of P225T/F/L, N230I, and H272Y/R/L in the succinate dehydrogenase iron sulfide gene (*SdhB*) (Veloukas et al. 2011; Yin et al. 2011). SBIs restrain fungal growth by inhibiting the synthesis of different enzymes during sterol synthesis. However, 3-ketoreductase (*erg27*) mutant strains that exhibit F412S/I/V mutations are resistant to SBIs (Debieu et al. 2013).

Compared with conventional methods, molecular detection methods are simpler, more sensitive, and efficient. These molecular methods include sequencing (Fillinger et al. 2012; Leroux et al. 2010), cleaved amplified polymorphic sequence (De Miccolis Angelini et al. 2014b), polymerase chain reaction (PCR) restriction fragment length polymorphism (Ishii et al. 2009; Van der Heyden et al. 2014), allele-specific PCR (De Miccolis Angelini et al., 2012, 2014a; Yin et al. 2012), tetra-primer amplification refractory mutation systems PCR (Muñoz et al. 2009), real-time PCR (Banno et al. 2008; Billard et al. 2012), and high-resolution melting analysis (Chatzidimopoulos et al. 2014). However, the majority of these methods are associated

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with some drawbacks. Their greatest weakness is that they can only detect a single codon of a single sample in turn. They are thus not suitable for large-scale resistance detection in *B. cinerea*. A simple and high-throughput assay for detecting fungicide resistance is required for large-scale monitoring of the resistant isolates of *B. cinerea*. The multiple detection method of the suspension array could simultaneously detect eight genotypes in four target genes (*BenA*, *SdhB*, *BcOS1*, and *erg27*) of *B. cinerea* that are associated with resistance to five fungicides (benzimidazoles or *N*-phenylcarbamates, DCs, SBIs, and SDHIs; Zhang et al. 2016). The eight genotypes included *BenA*-E198 (GAG), *BenA*-198A (GCG), *SdhB*-H272 (CAC), *SdhB*-272Y (TAC), *BcOS1*-I365 (ATC), *BcOS1*-365S (AGC), *erg27*-F412 (TTC), and *erg27*-412S (TCC).

In this study, we aimed to provide a flexible, high-throughput, and accurate method to obtain early warnings and monitor the fungicide resistance of *B. cinerea* isolates, which could provide the basis for selecting effective fungicides for controlling gray mold in the field.

Materials and Methods

Isolation of *B. cinerea* strains. A total of 257 single spore isolates from *B. cinerea* strains collected from tomato in Baoding, Cangzhou, Handan, Hengshui, Langfang, Qinhuangdao, Shijiazhuang, Tangshan, and Xingtai in Hebei Province in China were used in this study (Table 1).

DNA extraction. The isolates were cultured on potato dextrose agar (PDA) medium at 22°C in the dark for 14 days before DNA extraction. Mycelia were collected and frozen in liquid nitrogen, and the genomic DNA of *B. cinerea* was extracted with an Ezup Column Fungi Genomic DNA extraction kit (Sangon Biotech Co., Ltd., Shanghai, China). The DNA concentration was measured with a NanoDrop 1000 spectrometer (Thermo Scientific). All genomic DNA was diluted to 10 ng/μl for the subsequent experiment and was stored at -20°C until use.

Suspension array. The method of suspension array used here was reported by Zhang et al. (2016), including multiplex PCR, multiplex allele-specific primer extension (ASPE) PCR, hybridization, and detection. These steps were designed for amplifying target sequences of isolates in four fungicide resistance-associated genes (*BenA*, *SdhB*, *BcOS1*, and *erg27*) and attaching them to TAG labels. These products with TAG hybridized to specific microspheres with anti-TAG sequences. Genotypes of these isolates were analyzed with the Bio-Plex 200 system (Bio-Rad). The details are described below.

Multiplex PCR. Four DNA fragments of the target regions (*BenA*, *SdhB*, *BcOS1*, and *erg27*) were amplified simultaneously in one reaction. PCR reactions were conducted in a 25 μl reaction volume containing 10 ng of the isolate genomic DNA template, 1× PCR reaction buffer (-Mg²⁺), 1.5 mM of MgCl₂, 2 U of Platinum Taq DNA polymerase (Invitrogen), 0.2 mM of deoxy-nucleoside triphosphate (dNTP; TaKaRa), 0.05 μM of each *BenA* primer, 0.4 μM of each *SdhB* primer, and 0.2 μM of the other primers. The amplification conditions were as follows: 5 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 54°C and 30 s at 72°C; with a final extension at 72°C for 10 min. ExoSAP-IT (Affymetrix) was used to remove the unused primers and nucleotides and the PCR product was kept at 4°C until use. The primers used in multiplex PCR are detailed in Table 2.

Table 1. *Botrytis cinerea* isolates used in the study

Origin	Number of isolates	
	2016 (n = 84)	2017 (n = 173)
Baoding	24	58
Cangzhou	9	0
Handan	2	12
Hengshui	2	2
Langfang	6	20
Qinhuangdao	13	8
Shijiazhuang	15	43
Tangshan	10	19
Xingtai	3	11

Multiplex ASPE PCR. ASPE PCR was performed by coupling different TAG sequences to specific target genes of the ExoSAP-IT-treated PCR product. ASPE reactions were done in a final 20 μl volume containing 1 μl of 1× PCR reaction buffer (-Mg²⁺), 1.25 mM of MgCl₂, 0.75 U of Platinum Tsp DNA polymerase (Invitrogen), 25 nM of each TAG-ASPE primer, 5 μM of deoxyadenosine triphosphate (dATP), 5 μM of deoxythymidine triphosphate (dTTP), 5 μM of deoxyguanosine triphosphate (dGTP), and 5 μM of biotin-deoxycytidine diphosphate (biotin-dCTP; Invitrogen). Reaction mixtures were preheated for 2 min at 96°C; followed by 30 cycles at 94°C for 30 s, 55°C for 1 min, and 37°C for 2 min; with a final hold at 4°C until use. The primers used in multiplex ASPE PCR are detailed in Table 3.

Hybridization to MagPlex-TAG microspheres and detection. Fragments with the TAG sequence were hybridized with the MagPlex-TAG microspheres (Luminex, U.S.A.). The hybridization reaction was performed in a total volume of 50 μl containing 2,500 of each MagPlex-TAG microsphere, 5 μl of ASPE reaction product, 0.4 M of NaCl, 0.2 M of Tris, and 0.16% Triton X-100, pH 8.0. These mixtures were denatured at 95°C for 90 s and incubated at 37°C for 1 h. After incubation, samples were filtered through 1.2-μm MultiScreen HTS-BV 96-well filtration plates (Merck Millipore) and washed twice with 75 μl of 1× Tm hybridization buffer (0.2 M of NaCl, 0.1 M of Tris, and 0.08% of Triton X-100, pH 8.0). Microspheres with target DNA were then resuspended in 120 μl of 1× Tm Hybridization Buffer containing 0.1% bovine serum albumin and 2 mg/ml streptavidin-R-phycoerythrin (SAPE, Invitrogen), transferred to new 96-well plates (Corning), and incubated at 37°C for 15 min. Samples at 50 μl were then analyzed at room temperature on the Bio-Plex 200 system. For each sample, the median fluorescence intensity (MFI) of at least 100 beads was analyzed for each bead set.

Fungicides sensitive to carbendazim, diethofencarb, iprodione, and boscalid. Sensitivity of isolates to fungicides was determined by discriminating-dose and mycelial growth assays. Technical-grade carbendazim (98.3% active ingredient [a.i.]; Zhuozhou Huatai Fine-Chemical Factory), diethofencarb (95% a.i.; Shijiazhuang Pesticide Chemicals Factory), boscalid (95% a.i.; BASF), and iprodione (96.5% a.i.; Bayer Crop Science) were respectively dissolved in acetone to prepare stock solutions. Concentrations of 1 mg per liter of carbendazim, 5 mg per liter of diethofencarb, 5 mg per liter of iprodione, and 5 mg per liter of boscalid were used as a discriminating dose for the evaluations of the sensitivities of all 257 isolates in a simplified sensitivity test. Moreover, the half maximal effective concentration (EC₅₀) values of 103 randomly selected isolates were determined by mycelial growth assays. Fungicides were singularly added to PDA. Media containing serial concentrations of carbendazim (0, 1, 5, 10, 50, 100, and 500 mg per liter), diethofencarb (0, 0.1, 0.5, 1, 5, 10, and 50 mg per liter), iprodione (0, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 mg per liter), and boscalid (0.1, 0.5, 1, 5, 10, 50, and 100 mg per liter) were prepared. All isolates were precultured on PDA. Mycelial plugs (6 mm) from the edge of the 3-day-old colony on PDA were transferred to the medium containing fungicides. The orthogonal diameters were measured after 3 days at 25°C. Three replicates were used for each isolate. The resistance factor (RF) was calculated as the ratio between the EC₅₀ value of the resistant isolate and the mean EC₅₀ value of sensitive isolates (De Miccolis Angelini et al. 2014a). Resistant isolates to carbendazim, diethofencarb, and iprodione were defined as those with an RF > 5. The resistant isolates were classified as follows: lowly resistant (LR), with 5 ≤ RF < 10; moderately resistant (MR), with 10 ≤ RF < 40; and highly resistant (HR), with RF ≥ 40. In addition, isolates with RF values < 10 were considered sensitive to boscalid, and isolates were classified as follows: low resistant (LR), with 10 ≤ RF < 50; moderately resistant (MR), with 50 ≤ RF < 100; and highly resistant (HR), with RF ≥ 100.

Sequencing. As the standard of molecular detection of *B. cinerea*, sequencing was used to verify all mutations in the four genes in our study. Each pair of primers from multiplex PCR was used to amplify DNA from all 70 isolates of *B. cinerea*. The PCR products were sent to Sangon Biotech for sequencing.

Data analysis. For each allele, a criterion was set as the mean MFI of all blank controls ± 5 SD. The positive MFI values required to

meet this criterion determined the genotypes for each allele. The effective concentration of each fungicide that reduced mycelial growth by 50% (EC₅₀) was determined with DPS Data Processing System software.

Results

Resistance frequency of *B. cinerea* in Hebei Province. To monitor the overall status of fungicide resistance of *B. cinerea* in Hebei Province, a discriminating dose was used to test 257 isolates. The results showed that all isolates were resistant to carbendazim (Car^R), and resistance frequencies to diethofencarb (Die^R), iprodione (Ipr^R), and boscalid (Bos^R) were 92.08, 86.59, and 11.67%, respectively.

Detection of mutations using the suspension array. A total of 103 randomly selected *B. cinerea* isolates were genotyped by the Bio-Plex suspension array. The average MFIs of two replicates are shown in Table 4. The signal was considered positive when MFI was greater than the threshold. Otherwise, results with no positive signal in two alleles at one codon indicated that another mutation existed. For example, the genotypes of isolate 14 were *BenA*-E198# (where the number sign “#” indicates another mutant genotype), *SdhB*-H272, *BcOS1*-I365S, and *erg27*-F412. The genotypes of these isolates were directly obtained from the MFI value in the suspension array without subsequent analysis. Eight genotypes could be simultaneously detected by the suspension array from only one unit of the DNA template.

Molecular detection in *B. cinerea*. The results of the suspension array were analyzed and compared with the sequencing data. The results are presented in Table 5 as the amino acid substitutions of the field isolates. According to the sequencing results, the negative signal in the suspension array corresponded to other mutations, such as *BenA*-E198V, *BenA*-E198K, and *SdhB*-H272R. These genotypes were not set in the detection scope of the suspension array. For example, the genotype of isolate 14 in the *BenA* gene showed neither the wild type nor the E198A mutant, whereas the sequencing results showed that it was E198K at this codon. Therefore, other mutated genotypes of the measured codon in the suspension array did not affect the fungicide resistance decision. Using statistical analysis, the resistance frequency between the suspension array and sequencing was determined (Table 6). The other mutations in the detected codons of the suspension array showed no effect on the resistance frequency of *BenA*-198, *SdhB*-272, *BcOS1*-365, or *erg27*-412 (99.03, 4.85, 21.36, and 0%, respectively), and the results of these codons in the

suspension array and sequencing were fully consistent. The differences in resistance frequency between the two molecular methods were attributable to mutations in other codons that were not within the detection range of the suspension array, including *BenA*-200 and *BcOS1*-369+373. The resistance frequency of the *BenA*-F200Y and *BcOS1*-Q369P+N373S mutations was 0.97 and 54.37%, resulting in 100, 4.85, 75.73, and 0% resistance frequency in *BenA*, *SdhB*, *BcOS1*, and *erg27*, respectively. The differences in resistance frequency were mainly concentrated in the *BcOS1* gene associated with iprodione resistance; according to these results, the genotype of Q369P+N373S accounted for the majority of the resistance.

Multiple-fungicide resistance by molecular detection. According to the genotype results of *BenA*, *SdhB*, *BcOS1*, and *erg27*, 13 different genotypes are shown in Table 7. Isolates with multiple-fungicide resistance (up to four) represented the majority of the population (98.06%). Isolates with E198K or E198V mutation in *BenA* were resistant to both carbendazim and diethofencarb. Isolates with dual (Car^RDie^RBos^RIpr^RFen^S, Car^RDie^RBos^RIpr^RFen^S, Car^RDie^RBos^RIpr^RFen^S), triple (Car^RDie^RBos^RIpr^RFen^S, Car^RDie^RBos^RIpr^RFen^S), or quadruple (Car^RDie^RBos^RIpr^RFen^S) fungicide resistance accounted for 13.59, 83.50, and 0.97%, respectively, and type X (Car^RDie^RBos^RIpr^RFen^S) isolates accounted for the majority. All of the isolates had mutations in *BenA* conferring carbendazim resistance in field isolates of *B. cinerea*. For *BcOS1*, 67.74% of isolates (21 of 31) were mutant type in 2016, and the resistance frequency increased to 97.22% (70 of 72) in 2017. Only five isolates mutated in the *SdhB* gene were detected, and all isolates had no mutation in the *erg27* gene. One isolate with quadruple-fungicide resistance (Car^RDie^RBos^RIpr^RFen^S) was observed in this study.

The EC₅₀ distribution of isolates with different genotypes in Hebei. In addition to molecular detection, mycelial growth assays were used to detect fungicide sensitivity. Because fenhexamid was not used in these regions, sensitivity toward it was not detected. Four more frequently used chemical fungicides were selected: namely carbendazim, diethofencarb, boscalid, and iprodione. The relationships between different genotypes and their EC₅₀ distributions were analyzed (Fig. 1). As indicated in Table 8, the E198A, E198K, and E198V mutations were associated with high resistance, whereas one F200Y mutant was MR to carbendazim, with an EC₅₀ value of 1.09 mg per liter (Fig. 1A). The EC₅₀ values of isolates with E198V mutations were in the range of 0.57 to 4.97 mg per liter (Fig. 1B) and were MR or HR to diethofencarb. All isolates with

Table 2. Primers used in multiplex polymerase chain reaction (PCR)

Gene	Primer name ^a	Sequence (5'→3')	Primer concentration (μM)	PCR product size (bp)
<i>BenA</i>	<i>BenA</i> -F	GTCGTCCTCATCGCAAAGGT	0.05	353
	<i>BenA</i> -R	ACGGTGACAGCACGGAAGA	0.05	
<i>SdhB</i>	<i>SdhB</i> -F	ACACCGACCCAGCACCAGA	0.4	428
	<i>SdhB</i> -R	TTAGCAATAACCGCCAAA	0.4	
<i>BcOS1</i>	<i>BcOS1</i> -F	AGGTCACCCGCGTAGCAAGA	0.2	223
	<i>BcOS1</i> -R	TGCTTGATTTCACCCCTTACA	0.2	
<i>erg27</i>	<i>erg27</i> -F	GCGTGGAGAACTCTAAATCGG	0.2	191
	<i>erg27</i> -R	AGTGTAAGGCTTGATGGTATGC	0.2	

^a F = forward, R = reverse.

Table 3. Primers used in multiplex allele-specific primer extension polymerase chain reaction

Gene	Primer name	Sequences (5'→3') ^a
<i>BenA</i>	TAG-EAwt-F0	<u>CATCTTCATATCAATTCTCTTATTTGGTTGAGAACTCTGACGA</u>
	TAG-EA-F0	<u>ACAATATACATCACCTAACTTTCTTGGTTGAGAACTCTGACGC</u>
<i>SdhB</i>	TAG-HYwt-R0	<u>AACCTTCTCTCTATCTTATTTGAGCAGTTGAGAATAGTGTG</u>
	TAG-HY-R0	<u>AATTTCTTCTCTTTCTTTTCAAAATGAGCAGTTGAGAATAGTGTG</u>
<i>BcOS1</i>	TAG-ISwt-R0	<u>ATACTTTTCAAAACAAATAACACACTGCCCTGGACGCCTTCGA</u>
	TAG-IS-R0	<u>TCATCACCTTCTTACTTTACATTTGCCCTGGACGCCTTCGC</u>
<i>erg27</i>	TAG-FSwt-R0	<u>TTCAATTCAAATCAAAACACATCATCCATCCATCTTACAAGGTAGA</u>
	TAG-FS-R0	<u>ATCTCAATTACAATAACACACAAACATCCATCTTACAAGGTAGG</u>

^a TAG sequences are indicated by underlining.

E198K mutation showed high resistance to diethofencarb (Table 8). Mutant isolates with E198A and F200Y were diethofencarb sensitive (Die^S; Table 8). The EC₅₀ values of five resistant isolates with H272R mutation in *SdhB* ranged from 25.21 to 41.90 mg per liter (Fig. 1C) and were determined as LR to boscalid (Table 8). Ipr^R isolates with mutations at codons 365, 369, and 373 in *BcOSI* are shown in Figure 1D. We found a surprising result that one I365S mutant and one Q369P+N373S mutant were sensitive to iprodione. The EC₅₀ values of other resistant isolates with I365S mutations ranged from 1.14 to 5.96 mg per liter, and those with the Q369P+N373S mutation were

mainly concentrated in the range of 1 to 3 and 4 to 6 mg per liter. These isolates showed low resistance and moderate resistance to iprodione (Table 8). Ipr^R isolates with I365N mutations were MR (Table 8), with EC₅₀ values ranging from 3.40 to 7.38 mg per liter (Fig. 1D).

Sensitivity change to carbendazim, diethofencarb, iprodione, and boscalid. We further compared the EC₅₀ values of isolates collected in 2016 and 2017 (Fig. 2). Significant increments of EC₅₀ values against carbendazim (Fig. 2A) and iprodione (Fig. 2D) and a decrease of EC₅₀ values against diethofencarb (Fig. 2B) were observed from 2016 to 2017 in Hebei Province. Most isolates (95.15%) were

Table 4. Median fluorescence intensity (MFI) values of 10 representative field isolates^a

Sample no.	<i>BenA</i> -E198	<i>BenA</i> -198A	<i>SdhB</i> -H272	<i>SdhB</i> -272Y	<i>BcOSI</i> -I365	<i>BcOSI</i> -365S	<i>erg27</i> -F412	<i>erg27</i> -412S
1	45	24	210	18	42	397	998	45
4	275	26	187	21	1,420	46	1,162	24
8	30	322	20	28	953	37	777	46
14	21	39	168	28	36	313	1,000	31
15	27	16	337	21	1,832	42	1,494	57
18	19	29	302	28	570	25	905	35
23	32	33	253	29	1,351	35	1,164	25
53	362	48	200	33	900	40	832	45
74	46	43	24	25	32	53	2,006	62
93	296	38	23	26	51	45	743	79
ddH ₂ O ^b	53	65	50	40	42	56	89	83

^a Positive MFI values over critical value are highlighted in bold.

^b ddH₂O = double-distilled H₂O.

Table 5. Comparison of the Bio-Plex suspension array and sequencing in 10 representative results

Sample no.	Suspension array ^a				Sequencing					
	<i>BenA</i> -198	<i>SdhB</i> -272	<i>BcOSI</i> -365	<i>erg27</i> -412	<i>BenA</i> -198	<i>BenA</i> -200	<i>SdhB</i> -272	<i>BcOSI</i> -365	<i>BcOSI</i> -369+373	<i>erg27</i> -412
1	E198#	WT	I365S	WT	E198V	WT	WT	I365S	WT	WT
4	WT	WT	WT	WT	WT	F200Y	WT	WT	Q369P+N373S	WT
8	E198A	H272#	WT	WT	E198A	WT	H272R	WT	WT	WT
14	E198#	WT	I365S	WT	E198K	WT	WT	I365S	WT	WT
15	E198#	WT	WT	WT	E198K	WT	WT	WT	Q369P+N373S	WT
18	E198#	WT	WT	WT	E198V	WT	WT	WT	Q369P+N373S	WT
23	E198#	WT	WT	WT	E198V	WT	WT	WT	WT	WT
53	E198A	WT	WT	WT	E198A	WT	WT	WT	Q369P+N373S	WT
74	E198#	H272#	I365#	WT	E198V	WT	H272R	I365N	WT	WT
93	E198A	H272#	I365#	WT	E198A	WT	H272R	I365N	WT	WT

^a The number sign “#” indicates no calls; the Q369P mutations were always associated with N373S. WT = wild type.

Table 6. Resistance frequency of the isolates from the field using suspension array and sequencing

Gene	Codon	Genotype	Suspension array ^a		Sequencing		
			Number of isolates (n = 103)	Resistance frequency of target codon (%)	Genotype	Number of isolates (n = 103)	Resistance frequency of target codon (%)
<i>BenA</i>	198	E198	1	99.03	E198	1	99.03
		E198A	9		E198A	9	
		E198#	93		E198K	9	
					E198V	84	
	200	— ^b	—	—	F200	102	0.97
<i>SdhB</i>	272	H272	98	4.85	H272	98	4.85
		H272Y	0		H272Y	0	
		H272#	5		H272R	5	
<i>BcOSI</i>	365	I365	81	21.36	I365	81	21.36
		I365S	17		I365S	17	
		I365#	5		I365N	5	
	369+373	—	—	—	Q369+N373	47	54.37
<i>erg27</i>	412	—	—	—	Q369P+N373S	56	
		F412	103	0.00	F412	103	0.00
		F412S	0		F412S	0	

^a Four genotypes within detection range in suspension array were presented.

^b “—” indicated other codons outside the detection range in the suspension array.

sensitive to boscalid with EC₅₀ values ranging from 1.88 to 9.83 mg per liter, and only five isolates showed LR to boscalid. No significant change was observed in 2 years (Fig. 2C). The EC₅₀ values of Ipr^R isolates were 0.02 to 3.45 mg per liter in 2016 and ranged from 0.11 to 7.38 mg per liter in 2017 (Fig. 2D). The resistance frequencies to carbendazim, diethofencarb, iprodione, and boscalid were 100, 87.10, 3.23, and 61.29% in 2016 (*n* = 31) and 100, 91.67, 5.56, and 97.22% in 2017 (*n* = 72), respectively. These results indicate that the problem of multiple resistance to carbendazim, diethofencarb, iprodione, and boscalid is serious in Hebei Province.

The EC₅₀ variation of the isolates with different genotypes was further analyzed (Fig. 3). For carbendazim, the increment in fungicide resistance in Hebei (Fig. 2A) was attributed to the increments of EC₅₀ values with *BenA*-E198V and *BenA*-E198K mutations (Fig. 3A). The change of EC₅₀ in response to diethofencarb in different genotypes from 2016 to 2017 was not observed (Fig. 2B). The cause for the decrease in EC₅₀ distribution to diethofencarb in Hebei (Fig. 2B) was that the EC₅₀ values for *BenA*-E198K mutants were far greater than for *BenA*-E198V mutants, and the proportion of isolates that carried *BenA*-E198K to *BenA*-E198V reduced from 2016 to 2017 (Fig. 3B). The isolates that possessed *BcOSI*-I365S and *BcOSI*-Q369P+N373S mutations had increased EC₅₀ values. A new genotype of I365N with MR appeared in 2017 (Fig. 3C). These results accounted for the increment of iprodione EC₅₀ values from 2016 to 2017 (Fig. 2D).

Discussion

The development of *B. cinerea* multiple resistance to different classes of fungicides was reported previously (Bardas et al. 2010). Considering the large numbers of isolates that are resistant to a variety of fungicides, conventional methods have proved insufficient to provide adequate detection (Banno et al. 2008; Leroux et al. 2010; Van der Heyden et al. 2014). Our research group successfully developed a multiplex microbead-based suspension array for the simultaneous detection of eight alleles of four resistance genes using the Bio-Plex 200 platform (Zhang et al. 2016). The results of eight genotypes detected by suspension array were completely consistent with sequencing. The suspension array provides a high-throughput and high-efficiency method for molecular detection of fungicide resistance in field isolates.

In our study, the suspension array could detect the mutations at codon 198 in *BenA* except for the F200Y mutation, which was out of the detected range of this method. The percentage of isolates that mutated at codon 198 was 99.03% in Hebei Province. Previous studies also revealed that the mutations in codon 198 appeared more frequently (Luck and Gillings 1995; Ziogas et al. 2009). Therefore, the suspension array results are sufficient to detect the *B. cinerea* strains that are resistant to carbendazim and can finally guide carbendazim application in the field.

Ipr^R isolates detected in Hebei Province possessed point mutations at codons 365, 369, and 373 in *BcOSI*. The I365S and Q369P+N373S mutant strains accounted for the majority. Previous studies also found that mutations in the *BcOSI* gene associated with dicarboximide resistance are diverse in *B. cinerea* (Grabke et al. 2014; Ma et al. 2007). Considering the limitations of the detection scope in this article, further optimization of the suspension array method is required to cover a wider range, such as adding Q369P and N373S genotype detection in the *BcOSI* gene.

Isolates of *B. cinerea* resistant to benzimidazole fungicides were reported frequently in previous studies (Fan et al. 2017; Leroux et al. 2002; Liu et al. 2016). All isolates tested in this study were HR to carbendazim. Most of the isolates carried the E198V mutation in *BenA* (81.55%), which had a relatively low EC₅₀ for diethofencarb. The proportion of E198K mutants that were HR to diethofencarb was low (8.74%). Therefore, although the resistance frequency to diethofencarb was high, the average EC₅₀ was relatively low (6.01 mg per liter). Similarly, isolates had a HR frequency, but were LR or MR to iprodione. The I365S mutant with HR was not observed, which was reported in a previous study (Grabke et al. 2014). Increasing the dose of diethofencarb or iprodione might be useful to inhibit these resistant isolates mutated in *BenA* or *BcOSI*. Because *B. cinerea* was considered as a high-risk pathogen for the development

Fig. 1. Frequency distribution of half maximal effective concentration (EC₅₀) values for A, carbendazim, B, diethofencarb, C, boscalid, and D, iprodione. Data in the >500 position indicate that the EC₅₀ values for carbendazim were more than 500 mg per liter. Data in the >50 position indicate that the EC₅₀ values for diethofencarb were more than 50 mg per liter. The red dotted line represents 5 times the sensitivity baseline. R = resistance isolate, S = sensitive isolate, and WT = wild type.

Table 7. Different genotype of *Botrytis cinerea*

Multiple resistance	Type	Genotype				Number of isolates	
		<i>BenA</i>	<i>SdhB</i>	<i>BosI</i>	<i>erg27</i>	2016 (<i>n</i> = 31)	2017 (<i>n</i> = 72)
Single	Type I (Car ^R Die ^S Bos ^S Ipr ^S Fen ^S) ^a	E198A	WT ^b	WT	WT	2	0
Dual	Type II (Car ^R Die ^S Bos ^R Ipr ^S Fen ^S)	E198A	H272R	WT	WT	1	0
	Type III (Car ^R Die ^S Bos ^S Ipr ^R Fen ^S)	E198A	WT	I365S	WT	0	1
	Type IV (Car ^R Die ^S Bos ^S Ipr ^R Fen ^S)	E198A	WT	I365N	WT	0	1
	Type V (Car ^R Die ^S Bos ^S Ipr ^R Fen ^S)	E198A	WT	Q369P+N373S	WT	0	1
	Type VI (Car ^R Die ^R Bos ^S Ipr ^R Fen ^S)	E198V	WT	WT	WT	7	2
	Type VII (Car ^R Die ^S Bos ^S Ipr ^R Fen ^S)	F200Y	WT	Q369P+N373S	WT	1	0
Triple	Type VIII (Car ^R Die ^S Bos ^R Ipr ^R Fen ^S)	E198A	H272R	I365N	WT	0	3
	Type IX (Car ^R Die ^R Bos ^S Ipr ^R Fen ^S)	E198V	WT	I365S	WT	8	7
	Type X (Car ^R Die ^R Bos ^S Ipr ^R Fen ^S)	E198V	WT	Q369P+N373S	WT	6	53
	Type XI (Car ^R Die ^R Bos ^S Ipr ^R Fen ^S)	E198K	WT	I365S	WT	1	0
	Type XII (Car ^R Die ^R Bos ^S Ipr ^R Fen ^S)	E198K	WT	Q369P+N373S	WT	5	3
	Type XIII (Car ^R Die ^R Bos ^R Ipr ^R Fen ^S)	E198V	H272R	I365N	WT	0	1

^a Superscript letters R and S indicate resistance and sensitivity, respectively. Car = carbendazim, Die = diethofencarb, Bos = boscalid, Ipr = iprodione, Fen = fenhexamid.

^b WT = wild type.

of boscalid (Fan et al. 2017), the appearance of five H272R mutants indicated that boscalid should be used circumspectly in the future. According to these results, carbendazim is thus not recommended for controlling gray mold in Hebei Province in China. The application of diethofencarb, iprodione, and boscalid should be prudent. Rather, fenhexamid is suggested.

A high frequency of isolates with multiple resistance was observed in this study (98.06%). One isolate with quadruple resistance emerged in Hebei Province. Attention should be paid to prevent its spread under fungicide selection. Occurrence of multiple resistance was common in *Botrytis* isolates (Lu et al. 2016). The overuse of fungicides against *Botrytis* might result in poorer control of gray mold (Rupp et al. 2017). The frequency of multiple resistance strains increased, which was driven by the continuous use of fungicides (Kretschmer et al. 2009). Therefore, multiple-fungicide resistance should be monitored often to track the resistance status of *B. cinerea* in the field. The suspension array can satisfy the requirements of multiple-resistance detection and achieve the purpose of early warning.

We found that one genotype corresponds to one or more resistance levels through the combination of conventional and molecular

methods, which is consistent with previous studies (Grabke et al. 2014; Yin et al. 2011). We discovered that the level of resistance increased under the sustained fungicide application, even the strains carried the same mutation. Previous studies also reported that not only the resistance frequency but also the resistance level increased with continuous use of fungicides (Jia 2017). Molecular methods have been widely used in the study of fungicide resistance to detect the genotype of isolates (Banno et al. 2008; De Miccolis Angelini et al. 2014; Fernández-Ortuño et al. 2012). Molecular methods can provide information about the number of resistant isolates in the field. Resistance frequency, rather than resistance level, could be obtained by molecular methods. Traditional methods could be used to guide the application dose of fungicides. Therefore, the combination of two types of methods could more accurately reflect the resistance status in the field.

In this study, we also observed that two isolates harboring the I365S or Q369P+N373S mutation did not exhibit resistance to iprodione when tested by the mycelial growth assay. This might be attributable to the presence of heterokaryon, which may have multiple nuclei (Büttner et al. 1994; Faretra and Pollastro 1993). However, further research is needed to elucidate this observation.

Table 8. The number of isolates of *B. cinerea* in different genotypes corresponding to different resistance levels

Resistance level	Carbendazim				Diethofencarb				Boscalid		Iprodione			
	E198K	E198V	E198A	F200Y	E198K	E198V	E198A	F200Y	H272R	WT	I365N	I365S	Q369P+N373S	WT ^a
Sensitive	0	0	0	0	0	0	9	1	0	98	0	1	1	12
Low	0	0	0	0	0	0	0	0	5	0	0	9	29	0
Moderate	0	0	0	1	0	47	0	0	0	0	5	7	39	0
High	9	84	9	0	9	37	0	0	0	0	0	0	0	0

^a WT = wild type.

Fig. 2. Changes in half maximal effective concentration (EC_{50}) values for **A**, carbendazim, **B**, diethofencarb, **C**, boscalid, and **D**, iprodione in Hebei between 2016 and 2017. The red dotted line represents 5 times the sensitivity baseline. * $P < 0.05$, **** $P < 0.0001$ ($n = 103$). R = resistance isolate, S = sensitive isolate, and ns = no significant difference.

Fig. 3. Change trend of half maximal effective concentration (EC_{50}) values for **A**, carbendazim, **B**, diethofencarb, and **C**, iprodione in Hebei from 2016 to 2017. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The red dotted line represents 5 times the sensitivity baseline. ns = no significant difference, R = resistance isolate, S = sensitive isolate, and WT = wild type.

In summary, the molecular method of a suspension array can greatly simplify and speed up the process of multiple-resistance detection in field populations. Although the detection range needs to be further explored, the suspension array is recommended to first identify the resistance frequency and genotypes in large quantities of phytopathogen, which can achieve the goal of early warning, followed by the utilization of traditional methods to determine the fungicide sensitivity for the mutant isolates. Via this combination, more efficient and accurate results about multiple resistance of *B. cinerea* in the region could be obtained, so that fields could receive suitable fungicide treatments against *B. cinerea*, including the right fungicide types and fungicide dose.

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