Detection and Molecular Characterization of Resistance to the Dicarboximide and Benzamide Fungicides in *Botrytis cinerea* From Tomato in Hubei Province, China

M. Adnan, 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**, Key Lab of Horticultural Plant Biology, Ministry of Education, and College of Plant Science and Technology, Huazhong Agricultural University; and Pesticides Department, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt; **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

Altogether, 192 *Botrytis cinerea* isolates collected from tomato greenhouses at different locations in Hubei Province were evaluated for their sensitivity to fungicides procymidone and zoxamide. The mean effective concentration to cause 50% growth inhibition (EC_{50}) values of procymidone for sensitive and resistant isolates were 0.25 and 3.60 $\mu\text{g/ml}$, respectively. The frequency of procymidone-resistant (Pro^R) isolates was 18%, and the highest frequency was recorded in Jingmen. Positive cross-resistance was observed for Pro^R isolates to other dicarboximide fungicides but not to phenylpyrroles. Significant differences were observed for fitness parameters (i.e., mycelial growth, osmotic sensitivity, and virulence between sensitive and resistant isolates). Amino acid sequence of the *Bos1* gene revealed that Pro^R isolates carried either point mutations at codon 365 (I365S) or a pair of point mutations at codons 369 (Q369P) and 373

(N373S). For zoxamide, the mean EC_{50} values for sensitive and resistant isolates were 0.22 and 5.32 $\mu\text{g/ml}$, respectively. Approximately 14% of the isolates were found to be resistant to zoxamide, and the highest frequency of resistance was also observed in Jingmen. There was positive cross-resistance for zoxamide-resistant (Zox^R) isolates to carbendazim. No significant differences were observed for fitness parameters between zoxamide-sensitive and Zox^R isolates. Sequence analysis of the β -tubulin gene of *Botrytis cinerea* revealed two previously reported point mutations (E198A and E198K) and one new point mutation (T351I). This new mutation was detected in only those isolates which possessed the E198K but not E198A substitution. This study allows for a better understanding of the resistance development profile in Hubei Province. Results will be useful for the improvement of fungicide resistance management strategies.

Botrytis cinerea, the causal agent of gray mold, is a necrotrophic, airborne fungus and has a wide host range, including tomato (Dean et al. 2012; Kumari et al. 2014; Williamson et al. 2007). Control of *B. cinerea* is difficult due to its genetic diversity, wide host range, and survival as sclerotia, mycelia, and conidia (Braun and Sutton 1987; Diolez et al. 1995; Williamson et al. 2007). The fungus can cause preharvest losses up to 50% under favorable environmental conditions and losses may reach to 90% in severe conditions (Ellis and Grove 1982). The most efficient method to control *B. cinerea* is the chemical control, and about \$572 million worth of fungicides are sprayed annually to control gray mold (Dean et al. 2012).

Resistance developed after the usage of dicarboximide (DC) fungicides to control *B. cinerea* (Farettra and Pollastro 1991; Leroux et al. 1999). Excessive use of DC fungicides has resulted in decreased efficacy and resistance development throughout the world (Elad et al. 1992; Moorman and Lease 1992; Pappas 1997). Mutations in the *Bos1* gene are responsible for resistance to DC fungicides. Different point mutations in the *Bos1* gene were reported from resistant *B. cinerea* populations (Ma et al. 2007; Neumann 1992). A point mutation at codon I365 was predominant among *B. cinerea* isolates of different resistance phenotypes (Oshima et al. 2006). Point mutations always appeared in a pair in all procymidone-resistant phenotypes in commercially grown tomato (Liu et al. 2016). A combination

of two point mutations (Q369P and N373S) in the *Bos1* gene was responsible for moderate resistance (Grabke et al. 2014).

Zoxamide is a benzamide fungicide having a mode of action similar to that of benzimidazoles (MBCs). With high activity against oomycetes, it can also control true fungi such as *B. cinerea*, *Monilinia fructicola*, *Mycosphaerella fijiensis*, *Cercospora beticola*, and *Venturia inaequalis* (Cai et al. 2016; Young and Slawewski 2001). It controls the pathogen by inhibition of tubulin polymerization and arresting nuclear division after β -subunit microtubule binding (Young and Slawewski 2001). Numerous MBC-resistant isolates have been reported for *B. cinerea* all over the world (Stehmann and Waard 1996). The most effective method to control *B. cinerea* is chemical control but *B. cinerea* frequently develops resistance toward MBC fungicides (Lorenz 1988; Smith 1988). MBCs have been used to control gray mold for decades, including thiophanate-methyl, carbendazim, and others. Zoxamide is exceptional because it not only controls true fungi but also has good activity against oomycetes (Malandrakis 1979; Smith 1988). Resistance developed soon after MBCs were introduced but the situation is different in the case of zoxamide (Bi et al. 2011, 2014; Malandrakis et al. 2011; Young et al. 2001). In many previous studies, amino acid substitution in the β -tubulin (*TUB2*) gene appears to be the reason for benzimidazole's resistance in pathogens such as *B. cinerea*, *Penicillium expansum*, *Monilinia fructicola*, *Tapesia yallundae*, *T. acutiformis*, *Aspergillus nidulans*, and *Neurospora crassa* (Albertini et al. 1999; Cabañas et al. 2009; Dehne et al. 2004; Fujimura et al. 1992; Koenraadt et al. 1992; Ma et al. 2007; Orbach et al. 1986; Yarden and Katan 1993). Amino acid substitutions at codon 198 are frequently reported (Malandrakis et al. 2011). F200Y has also been reported as one more reason for high resistance to MBCs (Davidse and Ishii 1995; Yarden and Katan 1993). Low frequency of induced resistance was observed even after exposing them to UV radiations and increased zoxamide concentrations (Zhou et al. 2015). Zoxamide acts at a different *TUB2* site; this might be why a low frequency of zoxamide resistance was reported as compared with MBCs (Cai et al. 2015).

In this study, *B. cinerea* isolates collected from tomato were used to investigate the status of procymidone and zoxamide resistance

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

Funding: This work is supported by the Special Fund for Agro-Scientific Research in the Public Interest (numbers 201303023 and 201303025).

*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary tables are published online.

Accepted for publication 6 January 2018.

development. Key objectives of this study were to (i) assess the resistance frequency of *B. cinerea* isolates to procymidone and zoxamide, (ii) elucidate the molecular mechanism of procymidone and zoxamide resistance in *B. cinerea*, and (iii) provide clues for the management of resistant *B. cinerea* populations in Hubei to avoid unexpected control failures.

Materials and Methods

Fungicides. Fungicides used in this study included procymidone (50.0% active ingredient [a.i.], SUMILEX 50WP; Sumitomo Chemical Co., Ltd.), iprodione (50.0% a.i.; Qingdao Nintaus Pesticides Co., Ltd.), dimethachlon (96.0% a.i.; Wenzhou Pesticides Co., Ltd.), fludioxonil (97.0% a.i.; Hubei Jianyuan Chemical Co., Ltd.), technical-grade zoxamide (97.5% a.i.; Gowan Company, LLC), and technical-grade carbendazim (92.0% a.i.; Hubei Jianyuan Chemical Co., Ltd.). Stock solutions were prepared in suitable solvents as mentioned (Supplementary Table S1) and stored in the dark at 4°C till used.

Source of *B. cinerea* isolates. Single-spore *B. cinerea* isolates ($n = 192$) were recovered from diseased fruit or leaves of tomato grown at different commercial greenhouses. Sampling was done during 2012 and 2013 from eight different cities in Hubei Province (Supplementary Table S2). Isolates were preserved and stored at -20°C using the dried filter paper method described before (Luo et al. 2002). Isolates were identified as *B. cinerea* by a polymerase chain reaction (PCR)-based assay by using primer pair Bc-f+Bc-r (Fan et al. 2015).

Screening for resistance to fungicides. *B. cinerea* isolates were characterized as sensitive (S) or resistant (R) by using a discriminatory concentration (a concentration of fungicide that fully inhibits mycelial growth of the S isolates). According to previous studies, the discriminatory concentration was 5 $\mu\text{g/ml}$ for both procymidone and zoxamide (Cai et al. 2015; Moyano et al. 2004). Fungicides were dissolved in their respective solvents and added to potato dextrose agar (PDA) medium. The final concentration of acetone was limited to 0.1% (vol/vol), and the same concentrations of solvents were used as a control throughout the study. A plug (5 mm) of agar was taken from the borders of the 3-day-old colony grown at 23°C and transferred to the center of each PDA plate amended with the fungicides. After 3 days of incubation at 23°C , the growth of colony was observed visually. For each fungicide, the isolates which were able to grow on the control as well as on amended PDA were considered resistant while isolates which failed to grow on fungicide amended PDA were considered sensitive. In total, three replications were used, and the experiment was conducted twice.

Sensitivity of *B. cinerea* mycelial growth to procymidone and zoxamide. The sensitivity of procymidone-resistant (Pro^{R} ; $n = 35$) and zoxamide-resistant (Zox^{R} ; $n = 27$) isolates and randomly selected procymidone-sensitive (Pro^{S} ; $n = 20$) and zoxamide-sensitive (Zox^{S} ; $n = 20$) isolates was determined by evaluating the effective concentration to cause 50% growth inhibition (EC_{50}) values. Procymidone and zoxamide were dissolved in acetone and dimethyl sulfoxide, respectively. Solvents were used as a control, as previously described. To choose the effective concentrations for procymidone, first, a preliminary test was done to determine the range of effective concentrations (i.e., 0, 0.001, 0.01, 0.1, 1, and 10 $\mu\text{g/ml}$). Based upon preliminary tests, final concentrations used for procymidone EC_{50} estimation were 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 $\mu\text{g/ml}$ and for zoxamide were 0, 0.02, 0.06, 0.12, 0.36, 0.72, and 1.4 $\mu\text{g/ml}$. A plug (5 mm) of agar was taken from the borders of the 3-day-old fresh colony grown at 23°C and transferred to the center of PDA plates amended with a series of different fungicide concentrations. After incubation at 23°C for 3 days, average colony diameter was determined by measuring colony growth in two perpendicular directions, and the mycelial plug diameter (5 mm) was subtracted. Linear regression of percent inhibition in relation to control of mycelial growth versus $\log_{10}$ transformation of each concentration of procymidone and zoxamide was obtained. The EC_{50} was calculated for each isolate using linear regression in Data Processing System software (DPS, version 9.50). The EC_{50} values allowed the calculation of the resistance factor (the ratio of EC_{50} value for an R isolate relative to the mean EC_{50} value for S isolates

[RF value]), which shows the sensitivity level of isolates. Three replications for each isolate were used, and the experiment was repeated twice. Averages of results were calculated because there was no significant difference ($P > 0.05$) in mycelial colony diameter between the two experiments.

Cross-resistance. To determine the cross-resistance for Pro^{R} and Zox^{R} isolates to other fungicides, a discriminatory dose test was conducted according to previous studies (Alberoni et al. 2005; Li et al. 2003). Pro^{R} isolates were tested for cross-resistance with iprodione, dimethachlon, and fludioxonil but Zox^{R} isolates were tested only with carbendazim. According to previous studies, the discriminatory concentrations were 5, 1, 5, and 10 $\mu\text{g/ml}$ for dimethachlon (Ma et al. 2009), fludioxonil (Yin et al. 2015), iprodione, and carbendazim (Bardas et al. 2010), respectively. These fungicides were dissolved in their respective solvents. Briefly, a mycelial plug (5 mm) from the edge of a 3-day-old colony was transferred to amended or control PDA. After 3 days of incubation at 23°C , isolates which showed normal growth on amended PDA were considered resistant, whereas isolates which failed to grow were found to be sensitive. If resistant or sensitive phenotypes were consistent for two fungicides, cross-resistance was considered in these two fungicides. In total, three replications were used and the experiment was conducted twice.

Stability of R isolates. Stability of Pro^{R} and Zox^{R} isolates was investigated. The isolates were subcultured for 10 generations on fungicide-free PDA. EC_{50} was determined after 1st and 10th generation.

Fitness of R isolates to procymidone or zoxamide. Mycelial growth, osmotic sensitivity (procymidone), virulence, and sporulation were used as parameters to test the fitness of R isolates to procymidone or zoxamide. Sixteen procymidone (4 S, 4 low resistant [LR], 4 moderate resistant [MR], and 4 high resistant [HR]) and 10 zoxamide (1 S and 9 R) isolates were selected for this experiment.

A mycelial growth test was conducted on fungicide-free PDA. Mycelial plugs were cut from the borders of a 3-day-old colony and transferred to the center of PDA plates. The colony growth diameter was measured at two perpendicular directions at 60 h after incubation at 23°C in the dark.

Osmotic sensitivity was evaluated by transferring a 5-mm plug from a 3-day-old colony to PDA plates amended with a series of different NaCl concentrations (0, 1, 2, 4, 6, and 8%). Radial colony growth was measured after 3 days of incubation at 23°C .

Detached tomato fruit were used to evaluate sporulation fitness. Fresh fruit were washed with distilled water, then rinsed in 75% ethanol for 1 min followed by washing with distilled water, and placed on sterilized filter papers to dry. Plugs (5 mm) were taken from the margins of a 3-day-old colony and transferred to fruit punctured with a sterilized cork borer, as carried out in previous studies (Fan et al. 2016). The inoculated tomato fruit were incubated at 23°C and 100% humidity for 6 days, until sporulation occurred. Sterilized cotton buds were used to remove spores and placed inside 2-ml tubes containing distilled water with 0.05% Tween 20 to keep spores separated and prevent them from attaching to each other. Conidial suspension was then observed under a microscope.

A virulence test was conducted using fresh tomato. The fruit used were sterilized as described before. The concentration of spores was adjusted to $1 \times 10^6/\text{ml}$ and 10 μl of spore suspension was injected into fruit using a 100- μl syringe. Lesions area was measured with flexible plastic measuring tape after 4 days of incubation at 23°C and 100% humidity. Three fruit were used for each isolate. All experiments for fitness parameters were performed twice, with three replications each.

Efficacy of fungicides for control of *B. cinerea*. Procymidone and zoxamide fungicides were sprayed at the recommended field dose (1.66 and 0.003 g, respectively, per 500 ml of distilled water) onto detached, surface-disinfected tomato fruit and left until dry. Plugs (5 mm in diameter) were transferred from the border of a 3-day-old colony onto the surface of fruit punctured with a sterilized cork borer (Fan et al. 2016). Lesion area was measured with flexible plastic measuring tape after 5 days of incubation at 23°C . Three fruit were used for each isolate, and the experiment was done twice. Finally, efficacy was calculated using the following equation: disease

control efficacy = [(lesion growth of control – lesion growth of treatment)/lesion growth of control] × 100%.

DNA extraction. For DNA extraction, six to eight plugs taken from the corner of a 2-day-old colony were transferred to 50 ml of potato dextrose medium (200 g of potato and 20 g of dextrose per liter of water) in a flask and then incubated in a shaker (180 rpm) at 23°C for 3 days. Then, mycelia were dried and frozen. A sterilized mortar and pestle was used to grind mycelia in liquid nitrogen. Crushed tissue powder was collected in a 1.5-ml tube, DNA was extracted using Plant DNA Mini Kit DN15 (Beijing Aid Lab Biotechnologies Co. Ltd.). After DNA confirmation on an agarose gel, the concentration was measured by a NanoDrop (DNA trace measurement) machine (NanoDrop Technologies Inc.).

Amplification and sequencing of *Bos1* and *TUB2* genes. Based on the DNA sequence of gene *Bos1* (Gen-Bank accession number AF396827), BR1+BF1 and BR2+BF2 primer pairs were used to amplify two fragments (95 to 1,085 bp and 981 to 1,829 bp) of the *Bos1* gene (Table 1). A specific PCR primer pair (Bc365PM-F + Bc365PM-R) designed by using Primer Premier V5.0 was used for the remaining Pro^R isolates to amplify a fragment of the *Bos1* gene carrying the I365S single-point mutation (Table 1). The following PCR conditions were used: preheating at 94°C for 2 min; followed by 32 cycles of denaturation at 94°C for 30 s, annealing at 57°C for BF1+BR1 and Bc365PM-F + Bc365PM-R or 58.3°C for BF2+BR2 for 30 s, and extension at 72°C for 70 s; with a final extension of 72°C for 5 min.

For amplification of gene *TUB2*, primer pair P1-P2 was used to amplify the 1,765-bp fragment (Table 1). The 50-μl PCR volume included 2 μl of genomic DNA, 1 μl of each primer, 21 μl of double-distilled H₂O, and 25 μl of 2× Taq PCR Mix (AidLab Biotech). PCR conditions consisted of preheating at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 90 s; with a final extension of 72°C for 10 min and a holding temperature of 4°C.

Amplified PCR products were separated by electrophoresis on 1.5% agarose gels in Tris-acetate (Oshima et al. 2006) buffer and

photographed after staining with ethidium bromide. The fragment was purified using a UNIQ gel extraction kit (EasyPure Quick Gel Extraction Kit; TransGen Biotech Co.), then sequenced by Wuhan Tianyi Huiyuan Biotechnology Co., Ltd. BioEdit Sequence Alignment Editor version 7.1.3.0 and ClustalW were used for the sequence analysis.

Results

Frequency distribution of tomato R isolates. Of the 192 tomato isolates, 18% (35 isolates) and 14% (27 isolates) of the total isolates collected from the eight locations in Hubei were resistant to procymidone and zoxamide, respectively. Isolates from Jingmen showed the highest resistance frequencies (i.e., 56% for procymidone and 68% for zoxamide) for both fungicides. On the other hand, no R isolates were detected for procymidone in Xiangyang and zoxamide in Jingzhou and Shiyan (Fig. 1A and B).

Sensitivity of *B. cinerea* to procymidone and zoxamide. The EC₅₀ values for procymidone and zoxamide for S tomato isolates ranged from 0.08 to 0.67 μg/ml, with a mean of 0.25 μg/ml, and from 0.03 to 0.65 μg/ml, with a mean of 0.22 μg/ml, respectively (Fig. 2). The EC₅₀ values for 35 Pro^R isolates ranged from 1.27 to 9.36 μg/ml, with a mean of 3.60 μg/ml. The EC₅₀ values for 27 Zox^R isolate ranged from 1.97 to 18.02 μg/ml, with a mean of 5.32 μg/ml. Pro^R and Zox^R isolates showed a wide range of resistance, with RF values of 4.93 to 36.28, with a mean of 13.82, and 4 to 78.67, with a mean of 19.31, respectively (Table 2).

Different levels of resistance among Pro^R isolates were observed. Because different levels of resistance have been found associated with specific mutations in previous studies, the Pro^R phenotypes were categorized as LR, MR, and HR based on a previous study (Grabke et al. 2014). Among 35 R isolates, 8 isolates were LR, 21 isolates were MR, and 6 isolates were HR.

Cross resistance. All Pro^R isolates showed positive cross resistances with DC fungicides (i.e., iprodione and dimethachlon) whereas they were sensitive to a phenylpyrrole (PP) fungicide (i.e., fludioxonil).

Table 1. Primers used in this study

Prime name	Sequence (5'–3')	Location in gene	Reference
Bc-f (forward)	CAGGAAACACTTTTGGGGATA	Amplify 327-bp <i>Bos1</i> gene fragment of <i>Botrytis cinerea</i>	Fan et al. 2015
Bc-r (reverse)	GAGGGACAAGAAAATCGACTAA	...	...
BF1 (forward)	TACCGATCGAAAAACCCAAC	Amplify 990-bp (95–1,085) <i>Bos1</i> gene fragment of <i>B. cinerea</i>	Ma et al. 2007
BR1 (reverse)	TGGGCTGGTCTCTCAATCTT	...	...
BF2 (forward)	CAACGTTATGGCACAAAATCTCA	Amplify 848-bp (981–1,829) <i>Bos1</i> gene fragment of <i>B. cinerea</i>	...
BR2 (reverse)	AAGTTTCTGGCCATGGTGTTC	...	...
P1 (forward)	ATGCGTGAGATTGTATGTATTC	Amplify 1,765-bp β-tubulin gene fragment of <i>B. cinerea</i>	Liu et al. 2010
P2 (reverse)	CTATTCTCGCCCTCAATTG	...	...
Bc365PM-F (forward)	GAAAGCGAAGGCGTCCAG	Allele-specific polymerase chain reaction primers to detect I365S point mutation in <i>Bos1</i> gene	This study
Bc365PM-R (reverse)	GTCTCCCTTTGCGACAGC	...	...

Fig. 1. Frequency distribution of **A**, procymidone-resistant and **B**, zoxamide-resistant isolates of *Botrytis cinerea* collected from tomato greenhouses in eight locations of Hubei Province, China.

In the case of zoxamide, all R isolates showed positive cross resistances with a MBC fungicide (i.e., carbendazim) (Table 3).

Stability of R isolates. After 10 successive generations on fungicide-free PDA medium, mean EC_{50} values of Pro^R and Zox^R isolates were compared between the 1st and 10th generation. The variation of the mean EC_{50} values was not significantly different (procymidone, $P = 0.16$ and zoxamide, $P = 0.17$). Some isolates increased in resistance while others decreased, but collectively the isolates maintained the level of resistance (Table 4).

Fitness of R isolates. Selected Pro^R (S, LR, MR, and HR) and Zox^R isolates were evaluated for fitness parameters such as mycelial growth test, osmotic sensitivity, virulence, and sporulation. Significant differences were observed between Pro^S and Pro^R isolates regarding mycelial growth ($P = 0.002$), osmotic sensitivity ($P = 0.003$), and virulence ($P = 0.024$) but not in sporulation ($P = 0.867$). Zoxamide isolates showed no significant difference for all fitness parameters. In brief, significant differences were observed for mycelial growth between Pro^S and Pro^R isolates ($P = 0.002$) (Table 4). In the osmotic sensitivity test, Pro^R isolates showed slower and decreased growth on NaCl-amended PDA plates ($P = 0.003$). The percentage of inhibition of mycelial radial growth (PIMG) on PDA plates amended with 8% NaCl was the most apparent for R isolates compared with NaCl-free medium. Among R isolates, T400 showed the highest percentage of inhibition of PIMG compared with others. The results demonstrated that the R isolates were relatively more sensitive to osmotic stress compared with S isolates (Table 4). Virulence on tomato fruit, measured as lesion size, was also assessed between Pro^R and Pro^S isolates and Zox^R and Zox^S isolates. There were significant differences observed between mean lesion size of S and R isolates from procymidone ($P = 0.024$) and there was also significant difference found for virulence among different levels of procymidone resistance isolates (LR, MR, and HR) ($P = 0.059$). No significant difference was observed for virulence of Zox^S and Zox^R isolates. Upon comparison of sporulation of zoxamide isolates, no significant differences were found between S and R isolates ($P = 1.000$) (Table 4).

Control efficacy for R isolates. Pro^R and Zox^R isolates were subjected to an in vivo sensitivity assay for fungicides to assess the

Table 2. Sensitivity of *Botrytis cinerea*-resistant and -sensitive isolates to procymidone and zoxamide

Phenotype ^w	Isolate	Resistance ^x	EC_{50} ($\mu\text{g ml}^{-1}$) ^y	RF value ^z
Pro ^S	T227	S	0.13	...
Pro ^S	T401	S	0.15	...
Pro ^S	T402	S	0.17	...
Pro ^S	T429	S	0.17	...
Pro ^R	QJ4	LR	0.98	3.83
Pro ^R	T312	LR	2.37	9.19
Pro ^R	T405	LR	1.27	4.93
Pro ^R	T521	LR	1.43	5.55
Pro ^R	T79	MR	3.61	14.03
Pro ^R	T381	MR	3.65	14.18
Pro ^R	T399	MR	4.13	16.04
Pro ^R	T400	MR	4.15	16.10
Pro ^R	T177	HR	9.36	36.28
Pro ^R	T380	HR	5.41	20.98
Pro ^R	T387	HR	5.04	19.56
Pro ^R	T403	HR	6.92	26.83
Zox ^S	T507	S	0.16	...
Zox ^R	T295	R	3.32	14.50
Zox ^R	QJ40	R	0.92	4.03
Zox ^R	T121	R	2.42	10.57
Zox ^R	T135	R	2.52	11.03
Zox ^R	T388	R	4.72	20.62
Zox ^R	T396	R	2.85	12.46
Zox ^R	T400	R	10.83	47.31
Zox ^R	T403	R	1.50	6.53
Zox ^R	T405	R	18.01	78.66

^w Pro^S = procymidone sensitive, Pro^R = procymidone resistant, Zox^S = zoxamide sensitive, and Zox^R = zoxamide resistant.

^x Resistance levels of isolates. LR, MR, and HR represent low, moderate, and high resistance, respectively; S = sensitive and R = resistant.

^y EC_{50} = fungicide concentration resulting in inhibition of 50% mycelial growth.

^z RF = resistance factor, calculated by dividing the EC_{50} value of the resistant isolate by the mean EC_{50} value of the sensitive isolates.

Fig. 2. Frequency distribution of effective concentration to cause 50% growth inhibition (EC_{50}) values ($\mu\text{g/ml}$) for isolates of *Botrytis cinerea* sensitive and resistant to procymidone and zoxamide collected from tomato greenhouses in eight locations of Hubei Province, China. Pro^S and Pro^R represent procymidone-sensitive and -resistant isolates and Zox^S and Zox^R represent zoxamide-sensitive and -resistant isolates.

control efficacy on detached tomato fruit. Control efficacy for Pro^S and Zox^S isolates was 100% because all of them failed to grow or cause lesions on fruit. For Pro^R isolates, control efficacy was observed between 4.99 and 24.9%, and the lowest control efficacy was observed as 4.99% for isolate T177. In the case of Zox^R isolates, least control efficacy observed was 4.4% for isolate T405 (Table 4). The results showed that there was a significant difference ($P = 0.0002$) for control efficacy between S and R isolates of both procymidone and zoxamide fungicides, and the tested fungicides could not control the R isolates successfully, suggesting the high risk of management of these resistant *B. cinerea* isolates in practice.

Molecular characterization of *Bos1* and *TUB2* genes. Gene *Bos1* from one S, four LR, four MR, and three HR isolates to procymidone was sequenced. Sequence comparison of S and R isolates (4 LR and 3 HR) revealed a point mutation at codon 365 resulting in the substitution of isoleucine to serine (I365S) in R isolates. A pair of point mutations was detected in the sequence of four MR isolates. The first point mutation was observed at codon position 369 and the second point mutation occurred at codon 373, resulting in replacement of glutamine to proline (Q369P) and asparagine to serine (N373S), respectively. Amplification of a specific fragment of *Bos1* gene with a primer pair (Bc365PM-F + Bc365PM-R) detected a I365S point mutation in an additional six Pro^R isolates; two were LR while four were MR isolates.

Gene *TUB2* was sequenced from nine Zox^R isolates along with one Zox^S isolate. Sequence comparison of eight Zox^R isolates revealed a substitution at position 198 from GAG to AAG, resulting in replacement of amino acid from glutamic acid to lysine (E198K). The sequence of the remaining one R isolate revealed the substitution from GAG to GCG, resulting in replacement of amino acid at the same position from glutamic acid to alanine (E198A). Interestingly, all eight isolates with the E198K point mutation also possessed another point mutation which has not been previously reported. That is the substitution at amino acid position 351 from threonine to isoleucine (T351I).

For confirmation, an additional nine Zox^S isolates were sequenced, and none of them harbored this newly discovered point mutation but two isolates were found to possess the E198A point mutation. Both point mutations (E198K + T351I) appeared together in all selected isolates, while the new point mutation never combined with the E198A mutation (Fig. 3A and B).

Discussion

Fungicide resistance is a common problem occurring as a result of the extensive use of a fungicide to control a specific pathogen in the same area for several seasons. In Hubei Province, *B. cinerea* is a major factor limiting the production of soft fruit such as tomato. Recently, *B. cinerea* populations collected from different locations in Hubei Province developed resistance to several fungicides from different chemical groups, indicating that there is a need to design resistance management strategies to control the pathogen in Hubei Province (Fan et al. 2016, 2017). In this study, 18 and 14% isolates were found to be resistant to fungicides procymidone and zoxamide, respectively, which confirmed our hypothesis that fungicide resistance is becoming a serious problem in Hubei Province. Resistance to various classes of fungicides, including DC, PP, MBC, and benzamides, were previously reported not only in China but also around the world (Cai et al. 2016; Faretra and Pollastro 1991; Grabke et al. 2014; Korolev et al. 2011; Leroux et al. 2002; Liu et al. 2016; Moyano et al. 2004; Oshima et al. 2006; Petsikos-Panayotarou et al. 2003; Sun et al. 2010; Sergeeva et al. 2002).

The frequency of *B. cinerea* isolates resistant to DC was found 18, 20, and 23% at different locations in North and South Carolina (Grabke et al. 2014). The frequency of *B. cinerea* isolates resistant to procymidone in Jiangsu and Henan Provinces was recorded as 53 and 85%, respectively (Liu et al. 2016; Sun et al. 2010). Our results showed less frequency of resistant *B. cinerea* isolates as compared with these previous reports but we expect that it will increase with the extensive usage of the fungicides in the future.

Table 3. Cross-resistance test for procymidone and zoxamide with other relative fungicides in *Botrytis cinerea* isolates^w

Phenotype ^y	Isolate	Resistance ^z	Sensitivity ^x			
			Carbendazim	Dimethachlon	Iprodione	Fludioxonil
Pro ^S	T227	S	...	S	S	S
Pro ^S	T401	S	...	S	S	S
Pro ^S	T402	S	...	S	S	S
Pro ^S	T429	S	...	S	S	S
Pro ^R	QJ4	LR	...	R	R	S
Pro ^R	T312	LR	...	R	R	S
Pro ^R	T405	LR	...	R	R	S
Pro ^R	T521	LR	...	R	R	S
Pro ^R	T79	MR	...	R	R	S
Pro ^R	T381	MR	...	R	R	S
Pro ^R	T399	MR	...	R	R	S
Pro ^R	T400	MR	...	R	R	S
Pro ^R	T177	HR	...	R	R	S
Pro ^R	T380	HR	...	R	R	S
Pro ^R	T387	HR	...	R	R	S
Pro ^R	T403	HR	...	R	R	S
Zox ^S	T507	S	S	...	...	...
Zox ^R	T295	R	R	...	...	...
Zox ^R	QJ40	R	R	...	...	...
Zox ^R	T121	R	R	...	...	...
Zox ^R	T135	R	R	...	...	...
Zox ^R	T388	R	R	...	...	...
Zox ^R	T396	R	R	...	...	...
Zox ^R	T400	R	R	...	...	...
Zox ^R	T403	R	R	...	...	...
Zox ^R	T405	R	R	...	...	...

^w Discriminatory doses (a concentration of fungicide that fully inhibits mycelial growth of the sensitive isolates) used for sensitivity were 10, 5, 5, and 1 µg/ml for carbendazim, dimethachlon, iprodione, and fludioxonil, respectively.

^x S = sensitive and R = resistant, based on carbendazim sensitivity to zoxamide-resistant (Zox^R) isolates and dimethachlon, fludioxonil, and iprodione sensitivity to procymidone-resistant (Pro^R) isolates.

^y Pro^S = procymidone sensitive and Zox^S = zoxamide sensitive.

^z Resistance levels of procymidone-resistant isolates. LR, MR, and HR represent low, moderate, and high resistance, respectively.

Previous reports of fitness studies also reveal that there was a significant difference for DC but no significant difference between benzamide-sensitive and resistant field isolates of *B. cinerea*, which is in line with our findings (Liu et al. 2016; Moyano et al. 2004; Sun et al. 2010; Zhang et al. 2007). Fitness assessment suggested a high risk for the use of zoxamide because the R isolates have comparable contestability with S isolates in the field. Although DC-resistant isolates showed a certain fitness penalty, considering that the highest recommended rate of procymidone could not control them efficiently, the risk to use DC alone is still high to control *B. cinerea* isolates in the field. In our studies, *B. cinerea* isolates resistant to procymidone (DC) were sensitive to fludioxonil (PP), which is consistent with previous studies that reported no cross resistance of DC to PP for field isolates (Farettra and Pollastro 1993; Fillinger et al. 2012; Hilber et al. 1995; Leroux et al. 1999; Vignutelli et al. 2002; Ziogas and Kalamarakis 2001). This result indicated that fludioxonil is a good compound to control Pro^R *B. cinerea* populations.

Resistance to DC fungicide is related to mutations in the *Bos1* gene (Ma et al. 2007). *B. cinerea* isolates with various levels of resistance, whether they have a low, intermediate, or high range of EC₅₀, all possess one to several point mutations; and, among them, the I365 codon

mutation is predominant (Oshima et al. 2006). I365 N/S alone in the *Bos1* gene was responsible for low resistance (Grabke et al. 2014). Point mutation variations at single codon I365S/N/R of the *Bos1* gene were responsible for DC low resistance reported from France, England, Israel, Japan, New Zealand, Italy, Switzerland, and the United States (Cui et al. 2004; De Miccolis Angelini et al. 2007; Leroux et al. 2002; Ma et al. 2007; Oshima et al. 2002). Our results match with these reported studies of *Bos1* gene analysis. I365S point mutation was detected in LR isolates but, at the same time, this mutation was also detected in MR and HR isolates. The presence of this single point mutation in HR isolates may also be associated with multidrug efflux, as previously described (Grabke et al. 2014). Point mutations Q369P and N373S were always found together in Pro^R isolates and these results were consistent with the findings in Henan Province (Liu et al. 2016). It has been reported that a combination of two point mutations (e.g., Q369P and N373S) in the *Bos1* gene was responsible for moderate resistance (Grabke et al. 2014). Similarly, we detected the pair of point mutations Q369P and N373S from MR isolates. It should be noted that four MR isolates also possessed the I365S point mutation, suggesting that the MR phenotype is not associated with specific point mutation and different mechanisms should be involved.

Table 4. Fitness components, control efficacy, and stability of procymidone- or zoxamide-resistant *Botrytis cinerea* isolates from tomato, with sensitive isolates used as control[†]

Type ^t	Isolate	Level ^u	Growth ^v	PIMG (%) ^w	Vir (cm) ^x	Spor (× 10 ⁶ /cm ²) ^y	Efficacy ^z	Resistance stability EC ₅₀ (μg ml ⁻¹) ^s	
								1st	10th
Pro ^S	T227	S	6.97	98.44	2.20	6.91	100	...	...
Pro ^S	T401	S	6.51	96.13	2.77	4.06	100	...	...
Pro ^S	T402	S	6.51	83.61	2.48	5.56	100	...	...
Pro ^S	T429	S	7.00	95.63	2.84	5.26	100	...	...
Mean	...	S	6.75 ± 0.26 A	93.46 ± 3.42 C	2.57 ± 0.09 C	5.45 ± 0.33 A	100 ± 0.00 A	...	...
Pro ^R	QJ4	LR	5.30	104.23	2.37	7.75	8.80	0.98	1.12
Pro ^R	T312	LR	5.38	102.24	2.84	6.50	24.90	2.37	2.31
Pro ^R	T405	LR	6.12	108.08	3.01	6.50	11.73	1.27	2.24
Pro ^R	T521	LR	5.75	116.14	2.75	7.83	14.70	1.43	2.39
Mean	...	LR	5.64 ± 0.18 C	107.68 ± 4.84 B	2.75 ± 0.13 BC	7.15 ± 0.46 A	15.03 ± 2.47 B	1.51 ± 0.19 A	2.01 ± 0.27 A
Pro ^R	T79	MR	5.78	114.01	2.77	5.80	9.02	3.61	4.73
Pro ^R	T381	MR	5.60	109.06	3.13	6.84	7.70	3.65	2.88
Pro ^R	T399	MR	6.20	125.08	2.87	5.00	8.21	4.13	3.86
Pro ^R	T400	MR	5.92	134.58	2.91	5.13	9.91	4.15	4.53
Mean	...	MR	5.92 ± 0.09 BC	120.68 ± 6.14 A	2.92 ± 0.06 A B	5.69 ± 0.75 A	8.71 ± 2.19 BC	3.88 ± 0.28 A	4.00 ± 0.41 A
Pro ^R	T177	HR	5.81	114.59	3.08	6.75	4.99	9.36	7.55
Pro ^R	T380	HR	6.32	104.13	3.03	6.01	5.94	5.41	5.19
Pro ^R	T387	HR	6.63	104.47	2.91	8.86	13.80	5.04	4.99
Pro ^R	T403	HR	6.48	109.46	3.15	4.60	6.72	6.92	5.29
Mean	...	HR	6.31 ± 0.13 B	108.17 ± 4.34 B	3.04 ± 0.09 A	6.55 ± 1.07 A	7.86 ± 1.55 C	6.68 ± 0.46 A	5.75 ± 0.32 A
Zox ^S	T507	S	7.25 ± 0.21 A	...	5.17 ± 0.22 A	5.20 ± 0.58 A	100 ± 0 A	...	...
Zox ^R	T295	R	7.25	...	4.26	5.90	10.37	3.32	3.41
Zox ^R	QJ40	R	6.32	...	5.14	7.360	13.09	0.92	1.22
Zox ^R	T121	R	6.37	...	4.11	4.50	7.61	2.42	2.19
Zox ^R	T135	R	6.43	...	5.31	4.60	6.52	2.52	2.64
Zox ^R	T388	R	6.79	...	5.32	5.80	12.41	4.72	4.57
Zox ^R	T396	R	6.57	...	4.48	3.90	15.49	2.85	3.10
Zox ^R	T400	R	6.01	...	4.60	5.10	6.81	10.83	9.72
Zox ^R	T403	R	6.26	...	4.49	6.20	8.55	1.50	1.33
Zox ^R	T405	R	7.16	...	5.26	3.50	4.40	18.01	19.75
Mean	...	R	6.58 ± 0.12 A	...	4.78 ± 0.13 A	5.23 ± 0.33 A	9.47 ± 0.64 B	5.23 ± 0.17 A	5.32 ± 0.24 A

[†] Means were indicated as averages ± standard deviation; sensitive and the corresponding resistant isolates within a column followed by the same letter did not show significant differences according to the least significant difference test ($P = 0.05$).

^s EC₅₀ = mean effective concentration to cause 50% growth inhibition. Stability of procymidone-resistant (Pro^R) and zoxamide-resistant (Zox^R) isolates after 10 consecutive transfers on fungicide-free medium (1st and 10th transfer). Means were compared between 1st and 10th transfers of each group.

^t Phenotypes (Type); Pro^S = procymidone sensitive and Zox^S = zoxamide sensitive.

^u Resistance levels of isolates. LR, MR, and HR represent low, moderate, and high resistance, respectively; S = sensitive and R = resistant.

^v Mycelium growth was shown as colony diameter (in centimeters) at 60 h under 23°C.

^w Osmotic sensitivity 8%. Percentage of inhibition of mycelial radial growth (PIMG) was calculated using the formula $PIMG = [(C - N)/(C - 5)] \times 100$, where C is the colony diameter of the nontreatment control and N is that of the sodium chloride treatment.

^x Virulence (Vir) was shown as the diameter of lesion size (cm) at 3 days under 23°C.

^y Sporulation (Spor). Mean number (× 10⁶) of conidia per square centimeter.

^z 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.

Zoxamide is a benzamide fungicide having a mode of action similar to MBCs. It not only has good activity against oomycetes but also can control true fungi such as *B. cinerea* (Cai et al. 2016; Young and Slaweki 2001). Soon after MBCs were introduced, resistance started to develop, but this has not been observed for zoxamide (Bi et al. 2011, 2014; Malandrakis et al. 2011; Young et al. 2001). Mostly, amino acid substitution in the *TUB2* gene appears to be the reason of benzimidazole's resistance in pathogens such as *B. cinerea*, *P. expansum*, *M. fructicola*, *T. acutiformis*, *T. yallundae*, *A. nidulans*, *V. inaequalis*, and *N. crassa* (Albertini et al. 1999; Cabañas et al. 2009; Dehne et al. 2004; Fujimura et al. 1992; Koenraadt et al. 1992; Ma et al. 2007; Orbach et al. 1986; Yarden and Katan 1993). The risk of resistance development for zoxamide is very low whereas MBCs are at high risk for several pathogens (Albertini et al. 1999; Cabañas et al. 2009; Krämer and Schirmer 2007; Malandrakis et al. 2011; Sun et al. 2010; Smith 1988). In oomycetes, zoxamide lab-induced resistance is also rarely reported except in *Pythium sylvaticum* and *P. capsici* (Bi et al. 2011; Martinez et al. 2003). The difference between zoxamide and MBCs is that the zoxamide is used against diploid oomycetes whereas MBCs are against haploid stages of true fungi (Bi et al. 2014). Low risk for zoxamide resistance development was observed among eight *B. cinerea*-sensitive isolates; only one was mutated after many transfers, and the resulting mutants showed a fitness penalty. Molecular docking analysis revealed the formation of two strong hydrogen bonds between a side-chain of carbendazim and E198 but zoxamide did not form a direct interaction; this could be why mutations at this codon have less effect on the sensitivity of zoxamide compared with carbendazim. Authors suggested that the difference in the binding pocket of zoxamide and the target sites acting on *TUB2* makes it different from MBCs (Cai et al. 2015). In Liaoning Province and Inner Mongolia, 84% of *B. cinerea* isolates were found to be resistant to carbendazim (Cai et al. 2015). A high frequency (63.63%) of gray-mold-resistant isolates to carbendazim was also detected in Hubei Province (Fan et al. 2017). In our study, the frequency of *Zox^R* isolates was found to be just 14%, which is low and consistent with the previous studies. Such results suggest that zoxamide could be used as a candidate to manage the resistance to MBCs in *B. cinerea*.

Amino acid substitutions at codons 198 and 200 of the *TUB2* gene are frequently reported (Malandrakis et al. 2011). It has previously been noted that the replacement of amino acids from glutamic acid at codon 198 to alanine, valine, glycine, or lysine (A/V/G/K) is related to a high resistance level to carbendazim (Albertini et al. 1999; Cabañas et al. 2009; Fujimura et al. 1992; Koenraadt et al. 1992; Malandrakis et al. 2011). For carbendazim, the formation of two strong hydrogen bonds at codon 198 of the *TUB2* gene indicates

that it has a major role in resistance development (Cai et al. 2015). The most predominant point mutation found in *B. cinerea* isolates resistant to carbendazim was E198V (Fan et al. 2017). Point mutation E198V is not associated with zoxamide resistance (Cai et al. 2015; Dehne et al. 2004; Malandrakis et al. 2011). In our study, we were unable to detect E198V point mutation among the *Zox^R* isolates, indicating that the zoxamide is a good candidate to control MBC-resistant isolates with point mutation E198V. Mutation E198A of *TUB2* was detected in the majority of resistant *B. cinerea* isolates and has been responsible for high resistance to MBCs (Banno et al. 2008; Li et al. 2014). Increased sensitivity to zoxamide was observed due to E198A mutation (Cai et al. 2015; Dehne et al. 2004; Malandrakis et al. 2011). In our study, the E198A mutation was detected in one *Zox^R* isolate and two *Zox^S* isolates, suggesting that another mechanism instead of E198A conferred the resistance to zoxamide and the zoxamide could be used to control MBC-resistant isolates with point mutation E198A. In the previous study, point mutation M233I was suggested to be the determinant of zoxamide resistance (Cai et al. 2015). In our study, a new point mutation (T351I) was detected from eight of nine tested isolates. Molecular docking analysis of the *TUB2* gene with zoxamide and MBCs showed that even zoxamide was a component of binding pocket but had no direct interaction with E198. Codon E198 is strongly associated with MBC resistance (Cai et al. 2015). Interestingly, the newly detected point mutation T351I was only found in association with E198K in *Zox^R* isolates. Because E198K has no direct interaction with zoxamide, it is suggested that T351I might confer the resistance in these isolates. To the best of our knowledge, it is the first report of the T351I mutation in field isolates of *B. cinerea*.

Conclusively, the risk of fungicide development in *B. cinerea* is a serious problem for tomato growers in Hubei Province, China. According to the results of current study, we cannot rely on the use of DC fungicides separately to control this destructive disease. Integrated disease management is the strategy we should focus on, in addition to the use of biological control and agricultural practices. Using fungicides such as fludioxonil with a low risk of resistance development should be very important to manage the destructive *B. cinerea*. Zoxamide could also be used to control some MBC-resistant populations such as those with mutation E198V or E198A of *TUB2*.

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Fig. 3. Amino acid sequence alignment of *Botrytis cinerea* β -tubulin with mutation variations at positions 198 and 351. S represents sensitive isolate.

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