Genetics and Molecular Research

Detection and characterization of carbendazim resistance in *Sclerotinia sclerotiorum* isolates from oilseed rape in Anhui Province of China

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ABSTRACT. Stem rot caused by *Sclerotinia sclerotiorum* is a devastating disease of oilseed rape (*Brassica napus*) in Anhui Province of China. The fungicide carbendazim (methyl benzimidazole-2-yl carbamate; MBC) has been used to control this fungal disease since the 1980s. In the present study, 74 isolates of *S. sclerotiorum* from 13 regions of Anhui were collected, and the sensitivities of these isolates to MBC were examined to monitor fungicide resistance. We found that 22 of the 74 isolates showed resistance to MBC, indicating that *S. sclerotiorum* has developed resistance in parts of Anhui Province. PCR assays and DNA sequence analysis showed that isolates with high MBC resistance had a point mutation at position 198 in the β-tubulin gene that caused a glutamic acid-to-alanine change in the protein. The β-tubulin gene in low-resistance isolates did not have the mutation. These results indicate that the mutation in β-tubulin gene may be associated with high MBC resistance in *S. sclerotiorum.* The present study also found no correlation between MBC resistance and pathogenicity of *S. sclerotiorum* isolates, suggesting that the pathogenicity of *S. sclerotiorum*

isolates on oilseed rape did not vary with MBC resistance status.

Key words: *Sclerotinia sclerotiorum*; Carbendazim resistance; Molecular mechanism; Pathogenicity; Oilseed rape

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a cosmopolitan fungal pathogen that attacks more than 400 species of plants, including many important crops such as soybean, bean, sunflower, canola, and oilseed rape (Purdy, 1979; Boland and Hall, 1994; Zhang et al., 2014). Sclerotinia stem rot in rape as a consequence of *S. sclerotiorum* attack results in serious loss of yield and quality in oilseed rape (Boland and Hall, 1994; Xu et al., 2014). In recent decades, the disease has caused tremendous losses to rapeseed production in Canada (Bardin and Huang, 2001), U.S.A. (Purdy, 1979; Bolton et al., 2006), Australia (Letham et al., 1976) and China (Gao et al., 2014). Breeding programs for disease resistance are hampered by limited gene resources (Lu, 2003; Zhao and Meng, 2003). As a consequence, Sclerotinia stem rot continues to impose serious limitations on oilseed rape production all over the world. In practice, the application of fungicides is the principal response in most oilseed rape growing regions for managing Sclerotinia stem rot. The benzimidazole fungicide, carbendazim (methyl benzimidazole-2-yl carbamate; MBC), has been widely used to control this disease in China since the 1980s. However, the effectiveness of control achieved by the fungicide has declined continuously in some regions due to the development of MBC resistance (Shi et al., 2000; Zhang et al., 2003). Recently, MBC resistance in *S. sclerotiorum* has led to disease control failures; MBC resistance is widespread throughout Jiangsu Province with an estimated resistance frequency of 29.54% (Ma et al., 2009). It appears that the repeated and intensive applications of MBC have caused the emergence of resistant strains of *S. sclerotiorum* in fields in Jiangsu (Wang et al., 2014). However, the sensitivity of *S. sclerotiorum* isolates to MBC in Anhui Province is currently unknown. Thus, it is essential to investigate MBC sensitivity in *S. sclerotiorum* isolates from oilseed rape in Anhui Province in order to assess the risk of fungicide resistance.

In the present study, MBC resistance in *S. sclerotiorum* isolates from oilseed rape in the main growing areas of Anhui Province was measured and the resistance mechanisms were explored in an attempt to provide experimental evidence for the management of MBC resistance in *S. sclerotiorum* and the integrated control of Sclerotinia stem rot in rapeseed.

MATERIAL AND METHODS

Fungicides and media

Technical grade (98%) carbendazim (methyl benzimidazole-2-yl carbamate; MBC) was provided by Anhui Academy of Chemistry and Industry (Hefei, China) and was dissolved in 0.1 M hydrochloric acid (HCl) at 10 mg/mL as a stock solution.

Potato dextrose agar medium (PDA) was made from 200 g potato, 20 g agar and 20 g dextrose per liter of distilled water. Potato dextrose broth medium (PDB) was made from 200 g potato and 20 g dextrose per liter distilled water (Perez et al., 1992). These two media were used for routine culture and in the experiments to determine the sensitivity of *S. sclerotiorum* isolates to the fungicide.

Origin and collection of isolates

The isolates of *S. sclerotiorum* were collected from oilseed rape fields in Hefei, Tongcheng,

Jingde, Ningguo, Lujiang, Wuwei, Wuhu, Shouxian, Lu'an, Nanling, Xuanzhou, Fanchang, and Wangjiang of Anhui Province in China. The oilseed rape fields used for collection of the isolates were separated from each other by more than 10 km. In each field, several plants with symptoms of Sclerotinia stem rot were randomly collected, air-dried, placed in paper envelopes, and stored at -4°C . All isolates were derived from individual sclerotia collected from the oilseed rape plants. The sclerotia were surface sterilized in 0.1% sodium hypochlorite for 5 min, rinsed in sterile distilled water for 30 s, bisected and one of the two halves was placed on a PDA plate. PDA plates were incubated for 3 days at 25°C in a growth chamber (12 h photoperiod). Pure cultures were obtained by transfer of a single sclerotium and maintained on PDA slants at 4°C for 2 to 4 weeks. In all, 74 isolates were collected throughout Anhui Province.

Determination of sensitivity of *S. sclerotiorum* to MBC

The sensitivity of the *S. sclerotiorum* isolates to MBC was assayed using the colony growth rate method (Ma et al., 2009). A 5 mm mycelial plug was taken from the edge of a 3-day-old colony and placed onto the center of a PDA plate treated with 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 40, 80, 160, 320, or 640 mg/L MBC. For each isolate, three replicate plates per concentration were used. In the control group, distilled water at the same volume was added into the melted PDA and the mycelial plugs were cultured on the PDA plates. All the dishes were cultured for 2 days at 25°C in the dark. The cross method was employed to measure colony diameters at intervals of 24 h. The following formula was used to calculate the growth inhibition rate:

$$\cdot(\%) = \frac{(\text{control colony diameter - processed colony diameter})}{(\text{control colony diameter - 5})} \times 100$$

The growth inhibition rate was then transformed into a probability value of inhibition using the *Response Rate & Probability Value* conversion table and the MBC concentration was transformed into a logarithm (X) (Wang et al. 2014). The formula Y = a + b X was used to calculate the toxicity of MBC to *S. sclerotiorum* through the regression method. The EC_{50} value calculated from this formula was used to evaluate the degree of resistance of all colonies to MBC. The sensitivity of *S. sclerotiorum* isolates to MBC was classified into five types as suggested by Yang et al. (2004): sensitive (MBC^{S}), with EC_{50} < 0.5 mg/L; low resistance (MBC^{LR}), with an EC_{50} of 0.5-5 mg/L; medium resistance (MBC^{MR}), with an EC_{50} of 5-50 mg/L; high resistance (MBC^{HR}), with an EC_{50} of 50-500 mg/L; and super-high resistance (MBC^{VHR}), with EC_{50} > 500 mg/L.

The experimental data were analyzed by analyses of variance (ANOVA) using SAS GLM (SAS Institute, Inc., Cary, NC, USA). When the ANOVA was significant ($P < 0.05$), means were separated using Fisher's Protected Least Significant Difference (PLSD). Differences between the MBC resistant and MBC sensitive groups of isolates were analyzed using *t*-tests.

Mycelial culture

Eleven *S. sclerotiorum* isolates with different resistance levels were selected and cultured on PDA for 3 days: sensitive isolates FC, SX-11-1, and SX-A; low resistance isolates XZ, NGA15, SX-3, JDA13, and FD; and high resistance isolates SX4, SX2, and NGA5. Mycelia from each colony were transferred to 60 mL PDB and cultured for 6 days at 25°C . The mycelia were then collected with sterilized gauze and filters and washed twice in sterile water. Next, the mycelia were dried using a freezing vacuum dryer and stored at -20°C for later use.

Extraction of genomic DNA

Genomic DNA was extracted using the cetyltrimethylammonium bromide method (Stewart and Via, 1993), separated on a 1% agarose gel, and stored at -20°C for later use.

PCR amplification of β-tubulin gene

The following are sequences of the forward and reverse primers designed by Shanghai Invitrogen Biotechnology Co., Ltd. according to exon sequences of the *S. sclerotiorum β-tubulin* gene: forward primer ATGCGTGAGATCGTATGTATATCT and reverse primer: ACTAATGCAAGATCAGT AACCAGT. *β-tubulin* was isolated respectively from the genomic DNA of the 11 isolates with resistance to carbendazim using the forward primer and the reverse primer under following conditions: 94°C 5 min; 94°C 30s, 54°C 30s, 72°C 2 min, 35 cycles; 72°C 7 min; preserved at 4°C. The amplified products were sequenced after T-vector connection to confirm their identity.

Sequence analysis

DNASTAR was used for sequence analysis and DNAMAN was used to compare sequences. Information on sequences was obtained from GenBank (http://www.ncbi.nlm.nih.gov). The Homology of acquired sequences and known sequences were assessed using BLAST in order to identify mutations.

Test of pathogenicity of MBC-resistance isolates on oilseed rape

The pathogenicity of 74 isolates from different regions in Anhui was determined using the detached leaf inoculation test under greenhouse conditions (Liu et al., 2005). Wanyou 14 oilseed rape plants were grown in the greenhouse to the fourth true-leaf stage. Mycelia of *S. sclerotiorum* isolates were cultured on PDA for 3 days. Seven mm diameter agar discs were excised from the edges of growing colonies of each isolate and upended onto the detached leaves. Four replicate experiments were performed using eight leaves in each case. Treatment with blank agar discs was used as a control. All leaves were labeled and randomly arranged on wet gauze in containers that were collectively covered with transparent polyethylene bags. The leaves in the containers were incubated at 25°C in the dark. At 72 h after inoculation, the length and width of lesions were measured.

The isolates were separated into three types depending on the size of the disease spots on the leaves: strongly pathogenic, SPT, with an average diameter of disease spots >3cm after 72 h culture; weakly pathogenic, WPT, with an average diameter of disease spots <1 cm after 72 h culture; and intermediate pathogenicity, IPT, with an average diameter of disease spots between 1 and 3 cm after 72 h culture (Huang and Li, 2009).

RESULTS

Resistance of *S. sclerotiorum* from oilseed rape to MBC

Analysis of the sensitivities to MBC showed that the 74 isolates of *S. sclerotiorum* showed distinct differences in their EC_{50} values: 52 were sensitive to MBC with EC_{50} values <0.5 mg/L, 18 showed low resistance with EC_{50} values between 0.5 and 5 mg/L, 1 (SX11) had moderate resistance with an EC_{50} value = 25.0854 mg/L, and 3 (SX2, SX4 and NGA5) had high resistance

with EC_{50} values of 214.9593, 418.8048 and 88.5202 mg/L, respectively (Table 1). Resistant types accounted for 29.73% of the isolates: 24.32% were low resistance, 1.35% were moderate resistance, and 4.05% were high resistance types.

The results revealed a wide range of resistance to MBC in *S. sclerotiorum* isolates from the 13 growing areas with evidence of variation among regions. The SX population isolates from Shouxian showed the strongest resistance with EC_{50} values ranging from 0.0024 to 418.8048 mg/L, and a mean value of 73.4205 mg/L. The NG population from Ningguo also showed strong resistance with EC_{50} values ranging from 0.0010 mg/L to 88.5202 mg/L and a mean of 4.6544 mg/L. The least resistance was shown by the NL population from Nanling with EC_{50} values ranging from 0.0112 mg/L to 0.0335 mg/L and a mean value of 0.0224 mg/L (Table 2).

Table 1. EC_{50} values for MBC against *S. sclerotiorum* isolates from different regions of Anhui Province.

Isolate	EC_{50} (mg/L)	Isolate	EC_{50} (mg/L)	Isolate	EC_{50} (mg/L)
LA1	0.7642	JDB8	0.4445	NGA4	0.2161
LAH	0.5458	JDA12	0.3004	NGC8	0.0001
NL	0.0335	JDA11	0.3256	NGA15	1.2818
NL2	0.0112	JDA9	0.6816	NGB5	2.8745
XZ	1.7340	JDA16	0.0527	NGA1	1.2915
FC	0.0398	JDA15	0.0009	NGB-1-3	0.3810
HFD	0.5558	JDA7	0.0001	NGA6	0.0060
HF	0.4440	JDA17	0.0935	NGC6	0.0598
HFD-1-2	1.5575	JDA13	0.9210	NGA5	88.5202
HFD1	0.7627	XJDB6	0.5099	NGB1	0.0022
WH2	0.8595	XJDA14	0.0001	NGB-1-1	0.1886
WH	0.0974	XJDB8	0.5603	NGA8	1.0890
WW2	0.3552	XJDB7	0.0024	NGB6	0.0769
WWB8	0.5449	XJDB5	0.1957	NGA9	0.0813
WW	0.4882	SX-9-1	0.3287	NGC1	0.0010
WWD12	0.0232	SX-A	0.0501	NGC10	0.0479
WJC-2	0.0863	SX-3	1.0899	NGB4	0.0153
TCD2	0.1417	SX-5	0.4189	NGB1-2	0.2970
TC-7	0.0124	SX2	214.9593	LJA-5-2	0.0361
TC-10	0.4281	SX-11-1	0.0447	LJD-5-1	0.1144
TCC1	0.0029	SX6	0.0024	LJD-5-3	0.3970
TC-a-1	0.3183	SX4	418.8048	LJE-3-1	0.0033
TC-E-3	0.7314	SX11	25.0854		
TC-E-1	0.1652	NGC4	0.4911		
TC-15	0.0013	NGA7	0.8110		
JDA10	0.0785	NGC2	0.0105		

Table 2. The sensitivities of different populations of *S. sclerotiorum* to MBC.

Population	Origin	No.of isolates	EC_{50} range dimension (mg/L)	Average
JD	Jingde	15	0.0001-0.9210	0.2778
NG	Ningguo	21	0.0010-88.5202	4.6544
TC	Tongcheng	8	0.0013-0.7314	0.2252
SX	Souxian	9	0.0024-214.9593	73.4205
LJ	Lujiang	4	0.0033-0.3907	0.1377
WW	Wuwei	4	0.0232-0.5449	0.3529
HF	Hefei	4	0.4440-0.7627	0.8300
NL	Nanling	2	0.0112-0.0335	0.0224
WH	Wuhu	2	0.0974-0.8595	0.4785
LA	Liuan	2	0.5458-0.7642	0.6550
XZ	Xuanzhou	1	1.7340	1.7340
FC	Fanchang	1	0.0398	0.0398
WJ	Wangjiang	1	0.0863	0.0863

Geographic distribution of MBC-resistant isolates of *S. sclerotiorum*

We next examined the geographical distribution of the 22 resistant isolates across the

13 regions of Anhui province. The proportion of resistant isolates varied among the different populations. Both Lu'an isolates were resistant while resistance in isolates from Hefei, Wuhu, and Shouxian was present in 75, 50 and 44.44% of the samples, respectively. All isolates from Nanling, Xuanzhou, Fanchang, Wangjiang, and Lujiang were sensitive to MBC. The Ningguo isolates NGC8 and NGC1 were the most sensitive to MBC with EC_{50} values of 0.0001 mg/L and 0.0010 mg/L, respectively. However, another isolate from this region, NGA5, had high resistance to MBC. Overall, the rate of resistance was 4.76% in Ningguo. Two highly resistant isolates were identified in the sample from Shouxian (SX2 and SX4), accounting for 22.22% of the isolates collected from this region. Our results indicate that isolates of *S. sclerotiorum* from many regions have developed resistance to MBC, and that the level of resistance can vary within each area (Table 3).

Table 3. Survey of resistance in *S. sclerotiorum* populations and the proportion of isolates with resistance to MBC.

Isolate	Origin	No. of isolates	Resistant isolates	Sensitive isolate	Resistant proportion
JD	Jingde	15	4	11	26.67
NG	Ningguo	21	6	15	28.57
TC	Tongcheng	8	1	7	14.29
LJ	Lujiang	4	0	4	0
SX	Shouxian	9	4	5	44.44
WW	Wuwei	4	1	3	25.00
HF	Hefei	4	3	1	75.00
WH	Wuhu	2	1	1	50.00
NL	Nanling	2	0	2	0
XZ	Xuanzhou	1	0	1	0
FC	Fanchang	1	0	1	0
WJ	Wangjiang	1	0	1	0
LA	Lu'an	2	2	0	100.00
Total		74	22	52	28.00

β-tubulin gene sequences in MBC-resistant and MBC-sensitive isolates

A 1.6 kb fragment of the β-tubulin gene was amplified by PCR from MBC-sensitive and MBC-resistant isolates. Comparisons of the deduced amino acid sequences of the amplified products showed a point mutation in three high resistance isolates (NGA5, SX2, and SX4); in these isolates, the GAG at position 198 was replaced by GCG, resulting in an amino acid change from Glu to Ala. The β-tubulin gene in both the sensitive isolates and the low resistance isolates did not have the mutation (Figure 1). The results of our sequencing analysis indicated that mutation in the β-tubulin gene might be associated with MBC sensitivity in *S. sclerotiorum* isolates on oilseed rape in Anhui Province of Eastern China.

Pathogenicity of MBC-resistant isolates of *S. sclerotiorum*

Based on the results of our pathogenicity and MBC sensitivity tests, we examined MBC resistance and pathogenicity of the tested isolates on oilseed rape from different regions of Anhui (Table 4). We found that the three high resistance isolates, SX2 and SX4 were strongly pathogenic to oilseed rape (SPT), while NGA5 showed an intermediate level of pathogenicity (IPT). Furthermore, the moderate resistance SX11 isolate was a weakly pathogenic type (WPT). The MBC-sensitive isolates WW2, SX-5, and NGC6 varied in their pathogenicities and were SPT, IPT, and WPT, respectively. Similarly, the low resistance isolates LA1, HFD, and WH2 were WPT, IPT, and SPT, respectively. Thus, there was no correlation between MBC-resistance and pathogenicity in the *S. sclerotiorum* isolates tested (Table 4). The results suggest that the pathogenicity of *S. sclerotiorum* isolates on oilseed rape did not change with their MBC resistance.

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MB.SEQ  ATGCGTGAGATCGTTCATCTTCAAACCGGCCAATGTGGTAACCAAATTGGTGCTGCTTTC
SX2     ATGCGTGAGATCGTTCATCTTCAAACCGGCCAATGTGGTAACCAAATTGGTGCTGCTTTC
SX4     ATGCGTGAGATCGTTCATCTTCAAACCGGCCAATGTGGTAACCAAATTGGTGCTGCTTTC
NGA5    ATGCGTGAGATCGTTCATCTTCAAACCGGCCAATGTGGTAACCAAATTGGTGCTGCTTTC

MB.SEQ  TGGCAAACTATCTCTGGTGAGCATGGTCTTGACGGCTCTGGTGTCTACAATGGAACCTCC
SX2     TGGCAAACTATCTCTGGTGAGCATGGTCTTGACGGCTCTGGTGTCTACAATGGAACCTCC
SX4     TGGCAAACTATCTCTGGTGAGCATGGTCTTGACGGCTCTGGTGTCTACAATGGAACCTCC
NGA5    TGGCAAACTATCTCTGGTGAGCATGGTCTTGACGGCTCTGGTGTCTACAATGGAACCTCC

MB.SEQ  GATCTCCAACTTGAGCGTATGAACGTCTACTTCAACGAGGCTTCCGGCAACAAGTATGTT
SX2     GATCTCCAACTTGAGCGTATGAACGTCTACTTCAACGAGGCTTCCGGCAACAAGTATGTT
SX4     GATCTCCAACTTGAGCGTATGAACGTCTACTTCAACGAGGCTTCCGGCAACAAGTATGTT
NGA5    GATCTCCAACTTGAGCGTATGAACGTCTACTTCAACGAGGCTTCCGGCAACAAGTATGTT

MB.SEQ  CCCCGTGCCGTTCTCGTCGATTTGGAGCCAGGTACCATGGATGCCGTCCGTGCTGGTCCT
SX2     CCCCGTGCCGTTCTCGTCGATTTGGAGCCAGGTACCATGGATGCCGTCCGTGCTGGTCCT
SX4     CCCCGTGCCGTTCTCGTCGATTTGGAGCCAGGTACCATGGATGCCGTCCGTGCTGGTCCT
NGA5    CCCCGTGCCGTTCTCGTCGATTTGGAGCCAGGTACCATGGATGCCGTCCGTGCTGGTCCT

MB.SEQ  TTCGGTCAACTCTTCCGCCCAGATAACTTCGTTTTCGGTCAATCCGGTGCTGGTAACAAC
SX2     TTCGGTCAACTCTTCCGCCCAGATAACTTCGTTTTCGGTCAATCCGGTGCTGGTAACAAC
SX4     TTCGGTCAACTCTTCCGCCCAGATAACTTCGTTTTCGGTCAATCCGGTGCTGGTAACAAC
NGA5    TTCGGTCAACTCTTCCGCCCAGATAACTTCGTTTTCGGTCAATCCGGTGCTGGTAACAAC

MB.SEQ  TGGGCTAAGGGTCATTACACTGAGGGTGCTGAGCTTGTCGACCAAGTTCTTGATGTCGTT
SX2     TGGGCTAAGGGTCATTACACTGAGGGTGCTGAGCTTGTCGACCAAGTTCTTGATGTCGTT
SX4     TGGGCTAAGGGTCATTACACTGAGGGTGCTGAGCTTGTCGACCAAGTTCTTGATGTCGTT
NGA5    TGGGCTAAGGGTCATTACACTGAGGGTGCTGAGCTTGTCGACCAAGTTCTTGATGTCGTT

MB.SEQ  CGTCGTGAGGCTGAGGGCTGTGACTGCCTTCAAGGTTTCCAAATCACCCACTCTCTCGGT
SX2     CGTCGTGAGGCTGAGGGCTGTGACTGCCTTCAAGGTTTCCAAATCACCCACTCTCTCGGT
SX4     CGTCGTGAGGCTGAGGGCTGTGACTGCCTTCAAGGTTTCCAAATCACCCACTCTCTCGGT
NGA5    CGTCGTGAGGCTGAGGGCTGTGACTGCCTTCAAGGTTTCCAAATCACCCACTCTCTCGGT

MB.SEQ  GGTGGAACTGGTGCCGGTATGGGTACGCTTTTGATTTCCAAGATCCGTGAGGAGTTCCCA
SX2     GGTGGAACTGGTGCCGGTATGGGTACGCTTTTGATTTCCAAGATCCGTGAGGAGTTCCCA
SX4     GGTGGAACTGGTGCCGGTATGGGTACGCTTTTGATTTCCAAGATCCGTGAGGAGTTCCCA
NGA5    GGTGGAACTGGTGCCGGTATGGGTACGCTTTTGATTTCCAAGATCCGTGAGGAGTTCCCA

MB.SEQ  GATCGTATGATGGCTACCTTCTCCGTCGTCCCATCGCCAAAGGTTTCCGATACCGTCGTC
SX2     GATCGTATGATGGCTACCTTCTCCGTCGTCCCATCGCCAAAGGTTTCCGATACCGTCGTC
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Figure 1. Nucleotide sequences of the β-tubulin gene from three isolates of *Sclerotinia sclerotiorum* from oilseed rape in Anhui Province of China. The MBC resistant isolates SX2, SX5, and NGA5 have a point mutation at position 198 in which GAG is replaced by GCG (boxed sequence). The deduced amino acid sequence from the MBC resistant isolates show they have an amino acid change from Glu to Ala as a result of this mutation.

Continued on next page

Figure 1. Continued.

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SX4      GATCGTATGATGGCTACCTTCTCCGTCGTCCCATCGCCAAAGGTTTCCGATACCGTCGTC
NGA5     GATCGTATGATGGCTACCTTCTCCGTCGTCCCATCGCCAAAGGTTTCCGATACCGTCGTC

MB.SEQ   GAGCCATATAACGCTACTCTCTCTGTTCATCAATTGGTCGAGAACTCTGACGAGACCTTC
SX2      GAGCCATATAACGCTACTCTCTCTGTTCATCAATTGGTCGAGAACTCTGACGCGACCTTC
SX4      GAGCCATATAACGCTACTCTCTCTGTTCATCAATTGGTCGAGAACTCTGACGCGACCTTC
NGA5     GAGCCATATAACGCTACTCTCTCTGTTCATCAATTGGTCGAGAACTCTGACGCGACCTTC

MB.SEQ   TGTATCGACAACGAGGCTCTCTACGACATTTGCATGAGAACCTTGAAGCTCAGCCACCCA
SX2      TGTATCGACAACGAGGCTCTCTACGACATTTGCATGAGAACCTTGAAGCTCAGCCACCCA
SX4      TGTATCGACAACGAGGCTCTCTACGACATTTGCATGAGAACCTTGAAGCTCAGCCACCCA
NGA5     TGTATCGACAACGAGGCTCTCTACGACATTTGCATGAGAACCTTGAAGCTCAGCCACCCA

MB.SEQ   TCCTACGGAGATCTTAACCACTTGGTCTCCGCTGTCATGTCCGGTGTTACCACCTGTCTC
SX2      TCCTACGGAGATCTTAACCACTTGGTCTCCGCTGTCATGTCCGGTGTTACCACCTGTCTC
SX4      TCCTACGGAGATCTTAACCACTTGGTCTCCGCTGTCATGTCCGGTGTTACCACCTGTCTC
NGA5     TCCTACGGAGATCTTAACCACTTGGTCTCCGCTGTCATGTCCGGTGTTACCACCTGTCTC

MB.SEQ   CGTTTCCCTGGTCAACTTAACTCAGATCTCCGAAAGTTGGCTGTCAACATGGTTCCATTC
SX2      CGTTTCCCTGGTCAACTTAACTCAGATCTCCGAAAGTTGGCTGTCAACATGGTTCCATTC
SX4      CGTTTCCCTGGTCAACTTAACTCAGATCTCCGAAAGTTGGCTGTCAACATGGTTCCATTC
NGA5     CGTTTCCCTGGTCAACTTAACTCAGATCTCCGAAAGTTGGCTGTCAACATGGTTCCATTC

MB.SEQ   CCCCGTCTTCATTTCTTCATGGTTGGATTTGCTCCTTTGACCAGTCGTGGCGCACACTCT
SX2      CCCCGTCTTCATTTCTTCATGGTTGGATTTGCTCCTTTGACCAGTCGTGGCGCACACTCT
SX4      CCCCGTCTTCATTTCTTCATGGTTGGATTTGCTCCTTTGACCAGTCGTGGCGCACACTCT
NGA5     CCCCGTCTTCATTTCTTCATGGTTGGATTTGCTCCTTTGACCAGTCGTGGCGCACACTCT

MB.SEQ   TTCCGTGCTGTTACTGTTCCAGAGTTGACCCAACAAATGTATGATCCTAAGAACATGATG
SX2      TTCCGTGCTGTTACTGTTCCAGAGTTGACCCAACAAATGTATGATCCTAAGAACATGATG
SX4      TTCCGTGCTGTTACTGTTCCAGAGTTGACCCAACAAATGTATGATCCTAAGAACATGATG
NGA5     TTCCGTGCTGTTACTGTTCCAGAGTTGACCCAACAAATGTATGATCCTAAGAACATGATG

MB.SEQ   GCCGCTTCCGATTTCCGTAACGGTCGTTACTTAACCTGCTCTGCTATCTTCCGTGGTAAG
SX2      GCCGCTTCCGATTTCCGTAACGGTCGTTACTTAACCTGCTCTGCTATCTTCCGTGGTAAG
SX4      GCCGCTTCCGATTTCCGTAACGGTCGTTACTTAACCTGCTCTGCTATCTTCCGTGGTAAG
NGA5     GCCGCTTCCGATTTCCGTAACGGTCGTTACTTAACCTGCTCTGCTATCTTCCGTGGTAAG

MB.SEQ   GTTTCCATGAAGGAGGTTGAGGACCAAATGCGCAATGTCCAAAACAAGAACTCTTCCTAC
SX2      GTTTCCATGAAGGAGGTTGAGGACCAAATGCGCAATGTCCAAAACAAGAACTCTTCCTAC
SX4      GTTTCCATGAAGGAGGTTGAGGACCAAATGCGCAATGTCCAAAACAAGAACTCTTCCTAC
NGA5     GTTTCCATGAAGGAGGTTGAGGACCAAATGCGCAATGTCCAAAACAAGAACTCTTCCTAC

MB.SEQ   TTCGTCGAGTGGATCCCTAACAATGTCCAAACCGCCCTTTGCTCCATTCCTCCCCGTGGT
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Figure 1. Continued.

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SX2     TTCGTCGAGTGGATCCCTAACAATGTCCAAACCGCCCTTTGCTCCATTCCTCCCCGTGGT
SX4     TTCGTCGAGTGGATCCCTAACAATGTCCAAACCGCCCTTTGCTCCATTCCTCCCCGTGGT
NGA5    TTCGTCGAGTGGATCCCTAACAATGTCCAAACCGCCCTTTGCTCCATTCCTCCCCGTGGT

MB.SEQ  CTCAAGATGTCCTCCACCTTCGTCGGTAACTCGACCTCCATCCAAGAACTCTTCAAGCGT
SX2     CTCAAGATGTCCTCCACCTTCGTCGGTAACTCGACCTCCATCCAAGAACTCTTCAAGCGT
SX4     CTCAAGATGTCCTCCACCTTCGTCGGTAACTCGACCTCCATCCAAGAACTCTTCAAGCGT
NGA5    CTCAAGATGTCCTCCACCTTCGTCGGTAACTCGACCTCCATCCAAGAACTCTTCAAGCGT

MB.SEQ  GTCGGTGATCAATTCACTGCTATGTTCAGAAGAAAGGCTTTCTTGCATTGGTACACTGGT
SX2     GTCGGTGATCAATTCACTGCTATGTTCAGAAGAAAGGCTTTCTTGCATTGGTACACTGGT
SX4     GTCGGTGATCAATTCACTGCTATGTTCAGAAGAAAGGCTTTCTTGCATTGGTACACTGGT
NGA5    GTCGGTGATCAATTCACTGCTATGTTCAGAAGAAAGGCTTTCTTGCATTGGTACACTGGT

MB.SEQ  AGGGTATGGACGAGATGGAGTTCACTGAAGCTGAGTCCAACATGAACGATTTGGTCTCC
SX2     AGGGTATGGACGAGATGGAGTTCACTGAAGCTGAGTCCAACATGAACGATTTGGTCTCC
SX4     AGGGTATGGACGAGATGGAGTTCACTGAAGCTGAGTCCAACATGAACGATTTGGTCTCC
NGA5    AGGGTATGGACGAGATGGAGTTCACTGAAGCTGAGTCCAACATGAACGATTTGGTCTCC

MB.SEQ  GAGTACCAACAATACCAAGATGCCTCGATCTCTGAGGGAGAGGAGGAGTACGAAGAGGAA
SX2     GAGTACCAACAATACCAAGATGCCTCGATCTCTGAGGGAGAGGAGGAGTACGAAGAGGAA
SX4     GAGTACCAACAATACCAAGATGCCTCGATCTCTGAGGGAGAGGAGGAGTACGAAGAGGAA
NGA5    GAGTACCAACAATACCAAGATGCCTCGATCTCTGAGGGAGAGGAGGAGTACGAAGAGGAA

MB.SEQ  GCCCCAATTGAGGGCGAGGAATA
SX2     GCCCCAATTGAGGGCGAGGAATA
SX4     GCCCCAATTGAGGGCGAGGAATA
NGA5    GCCCCAATTGAGGGCGAGGAATA
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DISCUSSION

Oilseed rape is a major crop in China, and Anhui Province is one of the main production areas (Xu et al., 2014). Sclerotinia stem rot as a result of *S. sclerotiorum* infection is the principal disease of oilseed rape and has a significant impact on crop yields. In recent decades, MBC has been used widely for the control of this fungal disease; however, as resistance to this treatment has developed, it is important to assess the sensitivity of *S. sclerotiorum* to MBC in Anhui Province. Here, we examined 74 *S. sclerotiorum* isolates from 13 regions of Anhui Province and

Table 4. Resistance type and pathogenicity category of isolates of *S. sclerotiorum* from different regions of Anhui province.

Isolate	Resistance type[a]	Pathogenicity category[b]	Isolate	Resistance type[a]	Pathogenicity category[b]
NL	S	SPT	NGB-1-3	S	IPT
NL2	S	SPT	NGA6	S	SPT
XZ	S	SPT	NGC6	S	WPT
FC	S	SPT	NGB1	S	SPT
HF	S	IPT	NGB-1-1	S	IPT
WH	S	SPT	NGB6	S	SPT
WW2	S	IPT	NGA9	S	IPT
WW	S	SPT	NGC1	S	SPT
WWD12	S	IPT	NGC10	S	IPT
WJC-2	S	SPT	NGB4	S	IPT
TCD2	S	IPT	NGB-1-2	S	IPT
TC-7	S	WPT	LJA-5-2	S	IPT
TC-10	S	IPT	LJD-5-1	S	SPT
TCC1	S	SPT	LJD-5-3	S	IPT
TC-a-1	S	IPT	LJE-3-1	S	SPT
TC-E-1	S	SPT	LA1	LR	WPT
TC-15	S	SPT	LAH	LR	IPT
JDA10	S	SPT	HFD	LR	IPT
JDB8	S	SPT	HFD-1-2	LR	SPT
JDA12	S	SPT	HFD-1	LR	IPT
JDA11	S	SPT	WH2	LR	SPT
JDA16	S	SPT	WWB8	LR	SPT
JDA15	S	SPT	TC-E-3	LR	SPT
JDA7	S	SPT	JDA9	LR	SPT
JDA17	S	SPT	JDA13	LR	SPT
XJDA14	S	SPT	XJDB6	LR	SPT
XJDB7	S	SPT	XJDB8	LR	SPT
XJDB5	S	SPT	SX-3	LR	SPT
SX-9-1	S	SPT	NGA7	LR	IPT
SX-A	S	SPT	NGA15	LR	SPT
SX-5	S	IPT	NGB5	LR	IPT
SX-11-1	S	SPT	NGA1	LR	SPT
SX6	S	SPT	NGA8	LR	SPT
NGC4	S	IPT	SX11	MR	WPT
NGC2	S	IPT	SX2	HR	SPT
NGA4	S	IPT	SX4	HR	SPT
NGC8	S	SPT	NGA5	HR	IPT

(a) Type of MBC-resistance: S, sensitive; LR, low resistance; MR, moderate resistance; HR, high resistance. (b) Pathogenicity types: WPT, weak; IPT, intermediate; SPT, strong.

found resistance to MBC in 22 isolates (29.7%). To our knowledge, this is the first report of MBC resistance in *S. sclerotiorum* from Anhui Province of China. Although the current frequency of MBC^{MR} and MBC^{HR} isolates was low (5.41%), the rate of MBC^{LR} isolates was quite high (24.32%). Moreover, we also found that the pathogenicity of the isolates was not correlated with their MBC-resistance, suggesting that the pathogenicity of *S. sclerotiorum* isolates on oilseed rape does not change with MBC resistance. In other words, the MBC resistant isolates of *S. sclerotiorum* were as aggressive and fit as the original MBC sensitive isolates. In addition, we observed that the MBC resistant isolates showed comparable mycelial growth and sclerotia production as the sensitive isolates. This indicates that the MBC resistant isolates have strong reproductive fitness and have the capacity to develop into a dominant MBC resistant population in a field in a short time, which could lead to a failure in controlling Sclerotinia stem rot with MBC. Therefore, MBC resistance needs to be managed, and appropriate fungicide resistance management tactics need to be developed and employed, such as use of biological control agents, fungicide tank-mixing, or alternating MBC with other fungicides that have different modes of action; such tactics may aid the control of *S. sclerotiorum* on oilseed rape in Anhui.

Our investigation also indicated that the frequency of resistant isolates varied among populations, i.e. the resistant proportion varied considerably among sampled regions. The difference may be connected with the known variability in the genetic structure of *S. sclerotiorum* populations in fields and regions (Kohn et al., 1991). Most *S. sclerotiorum* isolates in the field reproduce through sexual propagation, and have the potential of hybridization (Atallah et al., 2004). Another reason might be differences in the MBC application level in different regions. According to our survey, MBC application levels were high in regions or fields with a high ratio or level of resistant isolates, such as Hefei and Shouxian. The MBC-resistant strains were detected following the frequent use of MBC over a long period of time. In these areas, different strategies for MBC resistance management need to be adopted.

Our analysis demonstrated that highly resistant isolates had a point mutation at position 198 of their β-tubulin gene sequence that caused the substitution of glutamic acid with alanine. The β-tubulin gene in low resistance and sensitive isolates did not contain this mutation. This indicates that the mutation in the β-tubulin gene might be associated with high MBC resistance in *S. sclerotiorum* isolates. Although a similar mechanism has been reported in other fungi, such as *Botrytis cinerea* (Yarden and Katan, 1993), *Venturia inaequelis* (Koenraadt et al., 1992) , *Aspergillus nidulans* (Jung et al., 1992) and *Neurospora crassa* (Koenraadt and Jones, 1993), this is the first report of its occurrence in *S. sclerotiorum* isolates on oilseed rape. As the β-tubulin gene has been fully cloned and sequenced, we can quickly detect the presence of highly resistant groups and monitor their development in the field by a specific PCR assay. This advance therefore offers a significant opportunity to improve the control of Sclerotinia stem rot and the management of MBC resistance in Anhui Province.

Conflicts of interest

The authors declare no conflict of interest.

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