Occurrence and Characterization of Dimethachlon Insensitivity in *Sclerotinia sclerotiorum* in Jiangsu Province of China

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ABSTRACT

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Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, is the main fungal disease of oilseed rape (*Brassica napus*) in China. Numerous fungicide applications are required for control. Dimethachlon, one of the dicarboximide fungicides, has been the major fungicide for disease control after benzimidazole resistance became widespread. Fungal populations were collected throughout Jiangsu Province between 2006 and 2007 in order to determine their sensitivity to dimethachlon. A total of 1,066 single-sclerotium isolates of *S. sclerotiorum* were collected, and most of the isolates were considered sensitive to dimethachlon. Five isolates collected in Yancheng and Changzhou showed normal growth at 5 µg/ml dimethachlon with the resistance factor ≈ 10 (resistance factor was estimated as ratios between the EC_{50} values of resistant isolates and the average EC_{50} values of sensitive ones) compared to the sensitive isolates (EC_{50} is the concentration of fungicide causing 50% reduction in growth). Through in vitro selection for resistance to the fungicide, 25 dimethachlon-resistant mutants were derived from 10 wild-type isolates of *S. sclerotiorum*. The resistance factors for the isolates ranged from 198 to 484, and the isolates were considered highly resistant to dimethachlon. Therefore, at least two different mechanisms of resistance seem to be involved: one that may provide a moderate resistance (insensitivity) and a second that may give a high resistance level under laboratory conditions. There was positive cross-resistance between dimethachlon and other dicarboximide fungicides, such as iprodione and procymidone, in these *S. sclerotiorum* isolates. The field dimethachlon-insensitive and the laboratory-induced dimethachlon-resistant isolates appeared to have mycelial growth, sclerotial production, and pathogenicity comparable to their wild-type parental isolates. Also, results of osmotic tests showed that there were no significant difference in mycelial radial growth between the field dimethachlon-sensitive and field dimethachlon-insensitive isolates on potato dextrose agar plates amended with 2, 4, 6, or 8% (wt/vol) NaCl, but the laboratory-induced dimethachlon-resistant isolates grew significantly more slowly than their wild-type sensitive parents under all concentrations of NaCl. Because these studies yielded a high frequency of laboratory resistance in *S. sclerotiorum*, together with the occurrence of field insensitivity, appropriate precautions against resistance development in natural populations should be taken.

Sclerotinia sclerotiorum (Lib.) de Bary is a cosmopolitan fungal pathogen that attacks more than 400 species of higher plants (7), including crops such as soybean, bean, sunflower, canola, and oilseed rape (*Brassica napus* L.) (37). Infection can occur on leaves, stems, and pods at different developmental stages (1,14,48). The disease is important in many countries, including Canada (4), the United

States (8,37), Australia (24), and China (51). For example, *Sclerotinia* stem rot in China causes yield loss ranging from 10 to 80%, and oil quality declines as well (3). Oilseed rape is the major oilseed crop in China, with approximately 70×10^6 ha in production (50). Jiangsu Province is the main production area, where oilseed rape is planted on about 700,000 ha every year.

Because breeding programs for disease resistance have been hampered by limited gene sources (28), *Sclerotinia* stem rot continues to impose serious limitations on oilseed rape production all over the world. In practice, application of fungicides is the principal tool in most oilseed rape crops for managing *Sclerotinia* stem rot. A benzimidazole fungicide, carbendazim (MBC), was routinely used to control this disease during the 1980s in China, but widespread control failures attributed to development of MBC resistance in *S. sclerotiorum* were reported in 2001 and 2002 (34,41,49).

The dicarboximide fungicides (e.g., iprodione, procymidone, and vinclozolin) have also been used for control of plant diseases incited by *S. sclerotiorum*, *S. homoeocarpa*, and *S. minor* for many years (9,19,21,32,33,44,45), although modes of action and resistance mechanisms of plant pathogens to these compounds are not yet well known. Dimethachlon (*N*-(3,5-dichlorophenyl)-succinimide, Ohric, Wenzhou Pesticide Factory, Zhejiang Province, China), a dicarboximide fungicide effective against various plant pathogenic fungi (36), has also been widely used for the control of *Sclerotinia* stem rot in China for more than 10 years, at an application rate of one to two sprays per season (26,42). However, it appears that repeated applications of these fungicides have caused the emergence of resistant strains of several plant pathogens, including *S. homoeocarpa* and *S. minor* (21,45), in other countries (5,22,23,30). Thus, it is necessary to incorporate effective antiresistance strategies when dicarboximide fungicides are extensively used to control plant diseases (10).

Previous studies also reported that high levels of dicarboximide resistance could be induced in the laboratory and that the laboratory-induced dicarboximide-resistant isolates exhibited greater osmotic sensitivity in several fungi with decreased virulence, while field isolates with dicarboximide resistance did not exhibit changed osmotic sensitivity and retained high virulence on plants (12,13,18,30), suggesting that the field-resistant isolates might have sufficient parasitic fitness to compete with field-sensitive isolates.

No information is available on dimethachlon sensitivity in field populations of *S. sclerotiorum* in China. The objectives of this study were to: (i) characterize dimethachlon sensitivity in several oilseed rape growing regions in Jiangsu Province; (ii) recover laboratory-resistant mutants of *S. sclerotiorum* to dimethachlon and investigate resistance stability in both the laboratory-induced dimethachlon-resistant isolates and the field dimethachlon-resistant isolates; (iii) explore the cross-resistance patterns between dimethachlon and other well-known dicarboximide fungicides, such as iprodione and procymidone; (iv) compare the biological characteristics of the isolates collected in the field with those of isolates resistant to dimethachlon and other dicarboximide fungicides obtained by selection in the laboratory; and (v) de-

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termine the osmotic sensitivity between field isolates resistant to dimethachlon and laboratory-resistant isolates.

MATERIALS AND METHODS

Fungal pathogens. All isolates of *S. sclerotiorum* used in the study were collected from oilseed rape fields in Jiangsu Province of China from 2006 to 2007 (Fig. 1). The oilseed rape fields were separated from each other by more than 10 km. In each field, several plants with symptoms of Sclerotinia stem rot were randomly collected from a designated 5.0 × 5.0 m² area, air-dried, placed in paper envelopes, and stored at -4°C. All isolates were derived from individual sclerotia collected from 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 potato dextrose agar (PDA; 200 g potato, 20 g agar, and 20 g dextrose per liter of distilled water) plate. The 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 (11,25,40).

Fungicides. Dimethachlon in technical grade (90.0%) was provided by Wenzhou Pesticide Factory and dissolved in methanol (>99.5%) to 10 mg/ml for the stock solution. Iprodione and procymidone in technical grade (96.2 and 98.0%, respectively) were provided by Jiangsu Kuaida Agrochemical Co. Ltd., China and Sumimoto Chemical Co. Ltd., Japan, respectively, and were dissolved in acetone to 10 mg/ml for the stock solution. All stock solutions were diluted into autoclaved media which had cooled to 40 to 50°C, and the pH was adjusted to 6.8 in all media using 0.1 mol/liter HCl.

Resistance determination. An initial qualitative assessment (preliminary test) was carried out for all isolates. To simplify the analysis of field resistance to dimethachlon in *S. sclerotiorum*, only two concentrations were used: 0 and 5.0 µg/ml. An inverted mycelial plug (5 mm in diameter) cut from the edge of a 3-day-old colony was transferred to a 9-cm petri dish containing amended or unamended PDA. After 2 days of incubation in a growth chamber at 25°C and 12-h photoperiod, mycelial growth was measured. Isolates that showed normal growth at 5 µg/ml dimethachlon were tentatively considered resistant (scored as growth), whereas the completely inhibited ones were considered sensitive (scored as no growth) (27).

Another series of tests was made to find the concentration of the fungicide causing a 50% reduction in the growth rate compared to an unamended control (EC₅₀). In the EC₅₀ assay, PDA medium was amended with six concentrations of dimethachlon: 0, 0.625, 1.25, 2.5, 5.0, and

10.0 µg/ml for all tentatively resistant isolates identified above and 0, 0.125, 0.25, 0.5, 1.0, and 2.0 µg/ml for 152 arbitrarily selected wild-type sensitive isolates in both 2006 and 2007 (72 isolates collected in 2006 and 80 isolates collected in 2007). Inverted mycelial plugs (5 mm in diameter) cut from the edges of 3-day-old colonies were transferred to 9-cm petri dishes containing the amended media. Three replicates per concentration were used, and all tests were repeated twice. The mycelial growth was measured after 2 days of incubation at 25°C in a growth chamber (12 h photoperiod). Two perpendicular diameters of each fungal colony were measured and averaged (the diameter of the plug was subtracted). EC₅₀ values were determined by probit analysis (2).

Sensitivity tests to find the minimum inhibitory concentration were carried out on the sensitive isolates collected in both 2006 and 2007. The procedure followed for the test for minimum inhibitory concentration was the same as described above for the EC₅₀ test, but different concentrations of the fungicide were used: 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 µg/ml among all the sensitive isolates. The evaluation was made after 2 days of incubation at 25°C in a growth chamber (12 h photoperiod). Fungal growth was observed, and the concentration that was able to inhibit mycelial growth completely (no mycelial growth) was recorded as the minimum inhibitory concentration. The test to find the minimum inhibitory concentration was repeated twice.

In vitro selection for resistance. To induce dimethachlon-resistance through selection, fresh mycelial plugs (5 mm in diameter) from colony margins of the five randomly selected wild-type sensitive isolates were transferred to PDA plates

amended with 5.0 µg/ml dimethachlon and incubated in the growth chamber for 3 days. Any fast-growing sectors from the otherwise restricted colonies were selected and transferred after 2 days to PDA plates amended with 5.0 µg/ml dimethachlon. Single-sclerotium isolates were established from dimethachlon-resistant mutants. Autoclaved PDA was amended with dimethachlon to obtain final concentrations of 15, 30, 60, 120, and 240 µg/ml, and the EC₅₀ values of resistant mutants were determined as described above.

The EC₅₀ values allowed the calculation of the resistance factor (RF), which shows the sensitivity level of isolates. The RF was estimated as ratios between the EC₅₀ values of resistant isolates and the average EC₅₀ values of sensitive ones. It was calculated both on the resistant isolates collected from the field for the 2 years of the monitoring work and on the resistant mutants selected under laboratory conditions for assessment of the resistance risk.

Resistance stability. All five field dimethachlon-resistant and three laboratory-induced dimethachlon-resistant isolates from each of their parental isolates (in all, 15 laboratory-induced dimethachlon-resistant isolates) were selected randomly, and 50 single-sclerotium isolates were established for each isolate as mycelial cultures. Each of the latter was transferred to PDA plates containing 5.0 µg/ml dimethachlon to determine whether the resistant isolates maintained dimethachlon resistance. In addition, all the resistant isolates and the three parental isolates were transferred eight times onto new fungicide-free PDA plates with three plates per isolate (2-day culture for one time) for over 16 days or stored on fungicide-free PDA slants with three slants per isolate at 4°C in the refrigerator for 60 days. The EC₅₀ val-

Fig. 1. Map of Jiangsu Province, China, where 1,066 isolates of *Sclerotinia sclerotiorum* were collected from oilseed rape plants with symptoms of Sclerotinia stem rot. The two cities shaded (Yancheng and Changzhou) indicate where field isolates with reduced sensitivity to dimethachlon were detected.

ues were then determined again as described above to test the resistance stability. The whole test to determine resistance stability was repeated twice.

Cross-resistance to other dicarboximide fungicides. All field dimethachlon-resistant isolates and six laboratory-induced dimethachlon-resistant isolates from each of the two parental isolates were subcultured on PDA plates. Cultures were grown for 2 days, and then 5-mm-diameter mycelial plugs were transferred from the margins of the colonies onto a series of concentrations of dimethachlon-, iprodione-, and procymidione-modified PDA media in 9-cm petri dishes, and incubated at 25°C (Table 1). The concentrations used were: 0.125-2, 0.625-10, and 18.75-300 µg/ml of dimethachlon; 0.075-0.4, 0.625-10, and 18.75-300 µg/ml of iprodione; and 0.075-0.4, 0.625-10, and 12.5-400 µg/ml of procymidione for the field sensitive isolates, the field dimethachlon-resistant isolates, and the laboratory-induced dimethachlon-resistant isolates, respectively (Table 1). The EC₅₀ values were determined as described above, and the sensitivity of mutants to dimethachlon and the other two fungicides was compared (47). The whole test to determine the cross-resistance was repeated twice.

Mycelial growth and sclerotial production by dimethachlon-resistant isolates. All the field isolates resistant to dimethachlon and six laboratory-induced dimethachlon-resistant isolates, together with their parental isolates, were chosen for the mycelial growth and sclerotial production test. Mycelial plugs (5 mm diameter) were cut from the edge of a 3-day-old colony and placed in the center of petri dishes (9 cm diameter) containing PDA. The plates were incubated at 25°C in a growth chamber (12 h photoperiod), and colony diameters (cm) were measured after 2 days of incubation. Three replicates were conducted for each of the isolates, and all the tests were repeated twice. Mean colony diameter (cm) was measured to compare mycelial growth. After 4 weeks, the numbers and the dry weight of sclerotia per plate were measured (35). Briefly, the number of sclerotia per plate was counted, the total dry weight of the sclerotia per plate was measured, and the average dry weight of a sclerotium was calculated. In addition, a fresh mycelial plug of each isolate from colony margins was transferred into a flask containing 100 ml

of potato dextrose (PD) broth for shake culture (120 r/min, 25°C) for 2 days. Then the mycelia were washed with distilled water three times to remove possibly contaminating medium and dried in an air drier at 80°C for 8 h. The dry weight of the mycelia was measured to further compare the mycelial growth. There were three replications for each of the isolates and the test was repeated twice.

In vitro pathogenicity tests. All the selected isolates used for the mycelial growth and sclerotial production test as described above were also used for the pathogenicity tests. Leaves from healthy oilseed rape plants cv. Ningyou 16 (Jiangsu Fengqing Seed Science Company) were detached, washed with sterile distilled water, blotted with sterile filter paper to remove excess water, and air-dried. A single leaf was detached from each plant. Leaf age, growing position, and leaf size were similar for all treatments in the tests. Mycelial plugs (5 mm diameter) from a 3-day culture of *S. sclerotiorum* were placed on the adaxial surface of the leaf (two per leaf, either side of the midrib, avoiding major veins). Lesions were measured as the mean of two diameters measured at right angles to one another after 4 days at 22°C under a 16-h photoperiod and ~80% relative humidity. The lengths of the long and short axes were averaged, and the radius was used to find the area of a circle (17). The experiment was performed twice with more than seven replicates per treatment.

Determination of osmotic sensitivity. For each isolate, a mycelial plug (5 mm diameter) was taken from the edge of a 3-day-old colony and transferred onto PDA amended with 2, 4, 6, or 8% (wt/vol) NaCl. Plates without NaCl were used as a nontreatment control. Three plates of each NaCl concentration were incubated at 25°C in a growth chamber (12 h photoperiod) for 2 days. The diameter of the fungal colony was measured in millimeters for each plate (30). The percentage of the mycelial radial growth inhibition (RGI) relative to the NaCl-free control was calculated. The experiment was repeated twice, and significant differences in colony diameter of each isolate were determined.

Statistical analysis. All analyses were conducted using SPSS 14.0 (Statistical Package for the Social Science, SPSS Inc., Chicago, IL). The EC₅₀ of the isolates, defined as the concentration of the fungicide that caused a reduction in colony

diameter of 50%, was estimated by linear regression of the log of the colony diameter versus the fungicide concentration. The ANOVA procedure of SPSS and Fisher's LSD, *P* = 0.05, were used to determine significant differences on the EC₅₀ values, the biological characteristics (mycelial growth, sclerotial production, and pathogenicity), and the osmotic sensitivity in field dimethachlon-sensitive, field dimethachlon-resistant, and laboratory-induced dimethachlon-resistant isolates.

RESULTS

Dimethachlon sensitivity of field isolates. Of the 1,066 single-sclerotium isolates analyzed in the present study, only five (CZ162, CZ138, YC310, YC312, and YC334) from Changzhou and Yancheng were insensitive to 5.0 µg/ml dimethachlon in the preliminary test (Fig. 1, Table 2). Moreover, mycelial growth of all sensitive isolates was completely inhibited at the concentration of 2.5 µg/ml, and thus 2.5 µg/ml could be treated as the minimum inhibitory concentration value for distinguishing the sensitive and resistant isolates in this study. The EC₅₀ values for inhibition of mycelial growth in the five field isolates insensitive to dimethachlon were 2.4 to 4.4 µg/ml, with an average EC₅₀ value of 3.2 µg/ml. The average EC₅₀ value for the 152 arbitrarily selected sensitive isolates was 0.3 µg/ml (Table 3). Therefore, the RF values for the five field isolates insensitive to dimethachlon were 7.1 to 13.2, with an average RF value 9.4.

In vitro selection for resistance. In the selection tests, all five parental sensitive isolates grew poorly on the PDA plates amended with 5 µg/ml dimethachlon during the first 3 days. Only a few spontaneous sectors from the five parental isolates grew rapidly after 2 days, and consequently, 25 resistant mutants from five parental sensitive isolates were obtained by selection on the fungicide after 10 days. The EC₅₀ values of six of the 25 mutants ranged from 68.0 to 166.4 µg/ml with the RF values 197.6 to 483.7 (Table 3), indicating high level of resistance.

Stability of dimethachlon resistance. All single-sclerotium progeny of insensitive field isolates and the resistant mutants grew normally on PDA amended with 5 µg/ml dimethachlon through eight transfers and when stored on PDA slants at 4°C for 60 days (data not shown).

Cross-resistance to dicarboximide fungicides. All five dimethachlon-in-

Table 1. Concentrations (µg/ml) used to determine the concentration causing 50% reduction in growth rate (EC₅₀) values for sensitivity to dicarboximides in *Sclerotinia sclerotiorum* isolates collected in Jiangsu Province, China

Resistance phenotypes of isolates	Dimethachlon (µg/ml)	Iprodione (µg/ml)	Procymidione (µg/ml)
Field dimethachlon-sensitive	2, 1, 0.5, 0.25, 0.125	0.4, 0.3, 0.2, 0.15, 0.1, 0.075	0.4, 0.3, 0.2, 0.15, 0.1, 0.075
Field dimethachlon-insensitive ²	10, 5, 2.5, 1.25, 0.625	10, 5, 2.5, 1.25, 0.625	10, 5, 2.5, 1.25, 0.625
Laboratory-induced dimethachlon-resistant	300, 200, 150, 75, 37.5, 18.75	300, 150, 75, 37.5, 18.75	400, 200, 100, 50, 25, 12.5

² Isolates that showed normal growth at 5 µg/ml dimethachlon were considered resistant, whereas the completely inhibited ones were considered sensitive.

sensitive field isolates and six laboratory-induced dimethachlon-resistant isolates had higher EC₅₀ values than the field sensitive isolates on the dicarboximide-amended PDA plates (Table 3). Comparison of the EC₅₀ between the insensitive and sensitive isolates showed that there was cross-resistance between dimethachlon and other well-known dicarboximide fungicides, such as iprodione and procymidone, in both field dimethachlon-insensitive and laboratory-induced dimethachlon-resistant *S. sclerotiorum* isolates (Table 3).

Mycelial radial growth and sclerotial production in vitro. The insensitive field isolates and the laboratory-induced dimethachlon-resistant isolates grew normally with thick aerial mycelium on PDA

plates amended with 5 µg/ml dimethachlon. The growth of the field sensitive parental isolates was completely inhibited. Most of the laboratory-induced dimethachlon-resistant isolates had reduced radial growth on PDA plates compared to their parental isolates (Table 4). Sclerotial production by the insensitive field and the laboratory-induced dimethachlon-resistant isolates and their parental isolates was compared from a 28-day culture on PDA. All isolates produced sclerotia, but numbers were reduced in the resistant compared to the sensitive isolates (Table 4).

Comparison of pathogenicity in vivo. Six laboratory-induced dimethachlon-resistant isolates and their parental isolates, and the 10 field-insensitive and -sensitive isolates exhibited pathogenicity on oilseed

rape leaves (Table 4). There were no significant differences in lesion area caused by most of the dimethachlon-resistant and -sensitive isolates. However, one field-insensitive isolate (YC310R) and two laboratory-induced dimethachlon-resistant isolates (CZ57R₂ and CZ57R₃) produced smaller lesions on oilseed rape leaves than the other isolates.

Comparison of osmotic sensitivity. The laboratory-induced dimethachlon-resistant isolates grew significantly more slowly than their wild-type sensitive parents on PDA plates amended with NaCl at each of the four concentrations, especially in the lower concentration (Fig. 2). However there were no significant differences ($P < 0.05$) in radial growth inhibition between the field-insensitive and -sensitive isolates at each of the NaCl concentrations.

Table 2. Number of isolates of *Sclerotinia sclerotiorum* sensitive and insensitive to dimethachlon found on oilseed rape growing in production areas throughout Jiangsu Province of China in 2006 and 2007

Cities	Years of dicarboximide use	Sample year ^y		Overall (by location) ^y
		2006	2007	
Suzhou	6	0 (33)	0 (70)	0 (103)
Taizhou	7	0 (96)	0 (70)	0 (166)
Wuxi	6	None ^z	0 (96)	0 (96)
Yangzhou	6	0 (40)	0 (67)	0 (107)
Huai'an	7	None	0 (47)	0 (47)
Yancheng	7	3 (50)	0 (58)	3 (108)
Suqian	7	None	0 (44)	0 (44)
Zhenjiang	7	None	0 (122)	0 (122)
Nantong	6	0 (122)	0 (89)	0 (201)
Changzhou	6	None	2 (103)	2 (103)

^y The number of field insensitive isolates is listed and the total number of tested isolates is given in parentheses. Isolates that showed normal growth at 5 µg/ml dimethachlon were considered resistant, whereas the completely inhibited ones were considered sensitive.

^z No samples were collected.

DISCUSSION

During the course of our monitoring study, which was carried out during the growing seasons of 2006 to 2007, sensitive isolates of *S. sclerotiorum*, together with a small subpopulation of isolates with reduced sensitivity to dimethachlon, were found in two cities (Yancheng and Changzhou, Fig. 1) of Jiangsu Province. Five *S. sclerotiorum* isolates with reduced sensitivity to dimethachlon were detected among 1,066 isolates tested.

Control failures that could be attributed to resistance to dimethachlon have not yet been detected from the oilseed rape fields in Jiangsu Province where dimethachlon has been extensively used for more than 6 years. However, high levels of resistance to

Table 3. Sensitivity of the selected isolates that showed different resistance levels to dimethachlon and the dicarboximide fungicides iprodione and procymidone in *Sclerotinia sclerotiorum*

Isolate ^x	Resistance phenotypes of the isolates	EC ₅₀ values of dimethachlon (µg/ml) ^y	EC ₅₀ values of iprodione (µg/ml) ^y	EC ₅₀ values of procymidone (µg/ml) ^y
CZ130	Field dimethachlon-sensitive	0.39 a ^z	0.21 a	0.19 a
CZ07	Field dimethachlon-sensitive	0.37 a	0.15 a	0.17 a
ZJ266	Field dimethachlon-sensitive	0.41 a	0.24 a	0.16 a
ZJ281	Field dimethachlon-sensitive	0.27 a	0.16 a	0.19 a
ZJ288	Field dimethachlon-sensitive	0.40 a	0.17 a	0.16 a
CZ94	Field dimethachlon-sensitive	0.38 a	0.18 a	0.16 a
CZ57	Field dimethachlon-sensitive	0.41 a	0.15 a	0.20 a
CZ87	Field dimethachlon-sensitive	0.32 a	0.27 a	0.26 a
YZ11	Field dimethachlon-sensitive	0.28 a	0.10 a	0.21 a
YZ12	Field dimethachlon-sensitive	0.37 a	0.16 a	0.12 a
HA84	Field dimethachlon-sensitive	0.23 a	0.21 a	0.22 a
YC312	Field dimethachlon-insensitive	4.42 c	4.56 d	3.47 b
YC310	Field dimethachlon-insensitive	2.37 b	2.25 b	4.17 b
YC334	Field dimethachlon-insensitive	2.99 b	2.00 b	2.48 b
CZ162	Field dimethachlon-insensitive	2.88 b	3.16 c	2.45 b
CZ138	Field dimethachlon-insensitive	3.12 b	3.00 c	2.61 b
ZJ288-1	Laboratory-induced dimethachlon-resistant	92.37 e	>100 f	220.39 e
ZJ288-2	Laboratory-induced dimethachlon-resistant	86.83 d	>100 f	182.81 d
ZJ288-3	Laboratory-induced dimethachlon-resistant	67.99 c	>100 f	462.54 g
CZ57-1	Laboratory-induced dimethachlon-resistant	166.40 h	>100 f	143.84 c
CZ57-2	Laboratory-induced dimethachlon-resistant	151.72 g	>100 f	515.19 h
CZ57-3	Laboratory-induced dimethachlon-resistant	124.81 f	42.40 e	276.88 f

^x ZJ288-1, ZJ288-2, ZJ288-3, CZ57-1, CZ57-2, and CZ57-3 were the laboratory-induced dimethachlon-resistant mutants derived from field dimethachlon-sensitive parental isolates ZJ288 and CZ57, respectively.

^y The EC₅₀ values, defined as the concentration of fungicide that caused a reduction in colony diameter by 50%, was estimated by linear regression of the log of the colony diameter versus the fungicide concentration.

^z Means followed by the same letter were not different according to Fisher's least significant difference (LSD) ($P = 0.05$).

dimethachlon could be obtained by in vitro selection for resistance. Based on our knowledge, this is the first report on the occurrence of dimethachlon-insensitive isolates of *S. sclerotiorum* in Jiangsu Province of China. Similar studies also showed that the heavy use of dicarboximide fungicides has led to the development of *S. homoeocarpa* (from golf courses) and *S. minor* (from peanut) isolates insensitive to these fungicides (21,45). However, another study reported that no dicarboximide-resistant isolates were detected from a field where dicarboximide fungicides were extensively used and losses had sometimes been severe (19).

Genetic analysis of dicarboximide resistance has demonstrated that the resistance is usually conferred by a single locus (*Daf1*) (12,13,15,29,30), although a second locus (*Daf2*) may also be involved in the resistance in laboratory-induced mutants (16). The disruption of *ubc1* gene and an enhanced level of antioxidant enzymes could also cause dicarboximide resistance (38,43,46). In light of recent studies on dicarboximide resistance, it was concluded that resistance mechanisms to dicarboximides in phytopathogenic fungi are complex (31). Consequently, it may be difficult to use molecular methods for detecting dicarboximide resistance in fungal populations (31). The presence of two resistance phenotypes of *S. sclerotiorum* in this study suggests that at least two different mechanisms of resistance may be involved: one that leads to a moderate resistance with RF ≈ 10 (as shown in the field dimethachlon-insensitive isolates) and another that leads to a high resistance with RF 197.6 to 483.7

(as shown in the laboratory-induced dimethachlon-resistant isolates). A recent study also reported two resistance phenotypes in *Stemphylium vesicarium* isolates from pear orchards that showed moderate resistance (RF ≈ 10) and high resistance (RF > 100) to dicarboximides, similar to the two resistance phenotypes in this study (2). Although the dicarboximide fungicides, such as iprodione, procymidone, and vinclozolin, have been widely used all over the world, the mode of action of these fungicides is still not fully understood (2,5,6,18,20,22,30,36,39). Dicarboximide fungicides are chemically different even if they have an analogous mode of antifungal action, which also supports the hypothesis

that there is more than one mechanism of resistance.

Information on fitness of resistant mutants is useful in developing effective antiresistance strategies. If fitness costs are associated with fungicide resistance, the frequency of the resistant pathogen population will decline when the fungicide selection pressure is discontinued in a field. A previous study reported that *S. minor* isolates from peanut with in vitro resistance to iprodione and vinclozolin were pathogenic to peanut in field microplots and survived as well as a fungicide-sensitive field isolate (9); whereas another study showed that the virulence of the dicarboximide-resistant *S. minor*

Fig. 2. Comparisons in osmotic sensitivity among laboratory-induced dimethachlon-resistant isolate CZ70R and its sensitive parent CZ70S, and field dimethachlon-insensitive isolate YC312R and other field dimethachlon-sensitive isolates YZ12S, YC24S, HA84S, YZ11S, and YZ36S. Data represent means $\pm$ SD of the two experiments since there were no significant ($P = 0.05$) differences between the two experiments.

Table 4. Comparison of mycelial growth, sclerotial production, and pathogenicity among field dimethachlon-sensitive, field dimethachlon-insensitive, and laboratory-induced dimethachlon-resistant isolates

Isolates ^v	Phenotype of sensitivity to dimethachlon	Mycelial growth		Sclerotia production		Lesion area (cm ²) ^y
		Mycelia radial growth (cm) ^w	Dry weight of mycelia (g) ^x	Number of sclerotia	Dry weight per sclerotium (mg)	
YZ12S	Field dimethachlon-sensitive	7.5 a ^z	0.65 a	25 a	2.2 b	41.8 a
YZ11S	Field dimethachlon-sensitive	7.4 a	0.65 a	24 a	2.2 b	43.0 a
ZJ281S	Field dimethachlon-sensitive	7.4 a	0.64 a	26 a	1.8 b	41.4 a
CZ57S	Field dimethachlon-sensitive	7.3 a	0.63 a	27 a	1.6 b	41.1 a
YZ36S	Field dimethachlon-sensitive	7.5 a	0.64 a	28 a	2.1 b	40.7 a
CZ138R	Field dimethachlon-insensitive	6.6 b	0.59 ab	9 b	6.0 a	45.3 a
CZ162R	Field dimethachlon-insensitive	6.5 b	0.53 ab	9 b	6.9 a	42.7 a
YC312R	Field dimethachlon-insensitive	6.4 b	0.53 ab	5 b	7.9 a	40.7 a
YC310R	Field dimethachlon-insensitive	6.5 b	0.60 ab	11 b	7.8 a	12.7 c
YC334R	Field dimethachlon-insensitive	6.6 b	0.50 ab	12 b	7.7 a	47.8 a
ZJ281R ₁	Laboratory-induced dimethachlon-resistant	6.5 b	0.50 ab	12 b	7.3 a	41.0 a
ZJ281R ₂	Laboratory-induced dimethachlon-resistant	6.6 b	0.42 b	11 b	7.4 a	50.4 a
ZJ281R ₃	Laboratory-induced dimethachlon-resistant	6.5 b	0.45 ab	9 b	7.2 a	40.7 a
CZ57R ₁	Laboratory-induced dimethachlon-resistant	6.7 b	0.55 ab	10 b	6.9 a	42.0 a
CZ57R ₂	Laboratory-induced dimethachlon-resistant	6.5 b	0.55 ab	8 b	7.2 a	22.8 b
CZ57R ₃	Laboratory-induced dimethachlon-resistant	6.4 b	0.53 ab	9 b	6.8 a	26.4 b

^v ZJ281R₁, CZ57R₂, CZ57R₃, CZ57R₁, CZ57R₂, and CZ57R₃ were the laboratory-induced dimethachlon-resistant mutants derived from field dimethachlon-sensitive parental isolates ZJ281 and CZ57, respectively.

^w The mycelial radial growth after 2 days of incubation on potato dextrose agar (PDA) at 25°C in a growth chamber (12 h photoperiod) was measured, and the two perpendicular diameters of each fungal colony were averaged.

^x A fresh mycelial plug of each isolate from colony margins was transferred into a flask containing 100 ml of potato dextrose (PD) broth for shake culture (120 r/min, 25°C) for 2 days, and the dry weight of the mycelia was measured to further compare mycelial growth.

^y Lesions were measured as the mean of two diameters measured at right angles to one another after 4 days at 22°C under a 16-h photoperiod and $\approx 80\%$ relative humidity.

^z Means followed by the same letter were not different according to Fisher's least significant difference (LSD) test ($P = 0.05$).

strains from lettuce declined over time (19). Also, laboratory-induced iprodione-resistant *Alternaria* isolates exhibited greater osmotic sensitivity than sensitive isolates, and this high osmotic sensitivity was considered to be responsible for lower pathogenicity and fitness of the resistant isolates than those of their sensitive counterparts (18,30).

During the course of this study, five field isolates of *S. sclerotiorum* were identified as having reduced sensitivity to dimethachlon, and these isolates showed comparable mycelial growth and pathogenicity to the wild-type sensitive isolates. The same case occurred in the laboratory-induced dimethachlon-resistant isolates. In addition, sclerotial production was lower in all laboratory-induced dimethachlon-resistant mutants and the field dimethachlon-insensitive isolates, indicating that the resistance mutation or mutations might presumably be involved in the reduced sclerotial production. We also found that these five field dimethachlon-insensitive *S. sclerotiorum* isolates did not show higher osmotic sensitivity than field dimethachlon-sensitive isolates, and they retained high virulence on oilseed rape. These results suggest that the field dimethachlon-insensitive isolates may have sufficient parasitic fitness to compete with the field dimethachlon-sensitive isolates in the field. However, laboratory-induced dimethachlon-resistant isolates had high levels of resistance to dicarboximide fungicides and showed higher osmotic sensitivity compared to their original sensitive parents.

In this study, both laboratory-induced dimethachlon-resistant isolates and field dimethachlon-insensitive isolates showed stable dimethachlon resistance/insensitivity, and there was positive cross-resistance between dimethachlon and other well-known dicarboximide fungicides in *S. sclerotiorum*. Because these studies yielded a high frequency of laboratory-resistance in *S. sclerotiorum* and a low frequency of field insensitivity, appropriate precautions against resistance development in natural populations should be taken. China now is facing the challenge of finding alternative fungicides to control *Sclerotinia* stem rot in oilseed rape crops. Moreover, in order to better understand dimethachlon resistance, the resistance mechanism in both laboratory-induced dimethachlon-resistant isolates and field dimethachlon-insensitive isolates is currently under further investigation in our laboratory.

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