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Sensitivity to iprodione and boscalid of *Sclerotinia sclerotiorum* isolates collected from rapeseed in China

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ABSTRACT

Baseline sensitivity of *Sclerotinia sclerotiorum*, causal agent of stem rot of rapeseed, to a dicarboximide fungicide iprodione was determined using 50 isolates (historic population) collected in 2001 from the rapeseed fields without a previous history of dicarboximide usage. The 50% effective concentration (EC_{50}) values to iprodione of these wild-type isolates ranged from 0.163 to 0.734 μg/ml with a mean of 0.428 μg/ml. In 2007 and 2008, 111 isolates (current population) were collected from rapeseed fields with 4–5 years of iprodione application. The EC_{50} values of these 111 isolates ranged from 0.117 to 0.634 μg/ml. The historic and current populations were not significantly ($P > 0.05$) different in sensitivity to iprodione. The EC_{50} values of these 161 isolates to a carboxamide fungicide boscalid ranged from 0.002 to 0.391 μg/ml with a mean of 0.042 μg/ml. In the laboratory, three iprodione-resistant (IR) isolates HA17-IR, SZ31-IR, and SZ45-IR were induced from wild-type isolates HA17, SZ31, and SZ45, respectively. The EC_{50} values of the IR isolates were 200-fold higher than those of the original wild-type parents. The IR isolates showed an increase in osmotic sensitivity. The IR isolate HA17-IR lost its ability to produce sclerotia, and showed a significantly lower virulence on rapeseed leaves than its parent isolate HA17. In contrast, the IR isolate SZ31-IR had a significantly higher virulence than its wild-type parent SZ31. PCR assays showed that the partial two-component histidine kinase (*os-1*) gene, which is the putative target gene of iprodione, was deleted in the low virulent IR isolate HA17-IR. DNA sequence analysis showed that each of the other two IR isolates SZ31-IR and SZ45-IR had two point mutations in their partial *os-1* genes. These results indicate that the mutations in *os-1* gene may be associated with dicarboximide sensitivity, sclerotial development, and virulence in *S. sclerotiorum*.

1. Introduction

The China rapeseed industry presently comprises over 7.2 million hectares, producing an annual crop valued at over 5 billion dollars. *Sclerotinia* stem rot of rapeseed (SSRR) caused by *Sclerotinia sclerotiorum* (Lib.) de Bary poses a major threat to the industry. *S. sclerotiorum* produces abundant large sclerotia which overwinter in the soil and crop residue, and could survive for 3–5 years. Under favorable environmental conditions, sclerotia at or near the soil surface germinate in spring to produce little mushroom-like spore producing structures called apothecia, from which several million ascospores could be released. The ascospores do not directly infect healthy green tissues of rapeseed plants, but initially infect rapeseed flowers or petals. When the petals fall, many will adhere to leaves and stems. Once infection is established on the fallen petals, the mold continues to grow into the stems and leaves under favorable weather conditions. If the main stems are infected, plants may die early and produce fewer or no seeds. New sclerotia are formed in infected stems to carry the pathogen over to the next season [1,2].

Sclerotinia sclerotiorum can infect more than 400 plant species, which enables the pathogen populations to persist from year to year. Because of the lack of highly resistant cultivars, the application of fungicides is the major approach for management of SSRR. The benzimidazoles thiophanate-methyl and carbendazim have been used widely in China for the control of SSRR for more than 20 years [3]. But a reduction of the efficacy of benzimidazoles has been observed in many fields because *S. sclerotiorum* populations have developed resistance to benzimidazoles [4]. Recently, the dicarboximide fungicides iprodione and dimethachlon have been introduced to control SSRR in China. Although resistance to iprodione has not been reported in field isolates of *S. sclerotiorum*, iprodione resistant fungal populations had been detected in *Botrytis cinerea* [5,6], *Monilinia fructicola* [7], *Sclerotinia homoeocarpa* [8], and *Sclerotinia minor* [9], which are genetically related to *S. sclerotiorum*. Thus, it is necessary to survey sensitivity of *S. sclerotiorum* populations for their sensitivities to iprodione, and asses the resis-

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tance risk for this compound in the control of SSRR before the appearance of any resistant isolates in field.

Boscalid, a fungicide in the carboxamide group, targets succinate dehydrogenase (SDH), which is a functional part of the tricarboxylic cycle and of the mitochondrial electron transport chain [10]. This fungicide has been used for the control of SSRR in the USA and European countries [11], but not currently registered for use on rapeseed in China. It therefore is a good time for establishment of baseline sensitivity of *S. sclerotiorum* to this fungicide before this compound is introduced into China.

The objectives of this study thus were to (i) establish the baseline sensitivity of *S. sclerotiorum* to iprodione and boscalid; (ii) test virulence of laboratory induced iprodione-resistant isolates; and (iii) investigate the possible mechanism of iprodione resistance in *S. sclerotiorum*.

2. Materials and methods

2.1. Isolate collections

Fifty isolates of *S. sclerotiorum* collected in 2001 (historic population) were used to determine baseline sensitivity of this fungus to iprodione since these isolates were obtained from rapeseed fields where iprodione and other dicarboximide fungicide have never been used before 2004 (Table 1). Another 111 isolates collected in 2007 and 2008 (current population) were obtained from the fields where iprodione had been applied once or twice per year since 2004. To obtain *S. sclerotiorum* isolates, mature sclerotia collected from infected stems of rapeseed plants were sterilized in 75% ethanol for 1 min and rinsed three times in sterilized water. Surface sterilized sclerotia were then cut into two pieces and placed on potato dextrose agar (PDA) (200 g potato, 20 g dextrose, 20 g agar, and 1 L water) plates amended with streptomycin at 0.5 g/L. After incubation of the plates at 25 °C for 3 days, a mycelial tip from each colony was transferred into a fresh PDA plate. From each sclerotium, only one isolate was recovered. The cultures were kept at 4 °C.

Table 1
List of *Sclerotinia sclerotiorum* isolates collected from rapeseed at different locations in different years used in this study.

Origin (City, Province)	Number of isolates	Year of isolation
Baoying, Jiangsu	4	02/2001
Changshu, Jiangsu	6	02/2001
Guanyun, Jiangsu	1	02/2001
Haian, Jiangsu	5	02/2001
Haimen, Jiangsu	1	02/2001
Huai'an, Jiangsu	2	02/2001
Jianhu, Jiangsu	4	02/2001
Jinhu, Jiangsu	1	02/2001
Jurong, Jiangsu	2	02/2001
Liyang, Jiangsu	4	02/2001
Nanjing, Jiangsu	8	02/2001
Rugao, Jiangsu	1	02/2001
Tianchang, Anhui	1	02/2001
Tongzhou, Jiangsu	1	02/2001
Wujin, Jiangsu	6	04/2001
Xinghua, Jiangsu	1	02/2001
Xishan, Jiangsu	1	02/2001
Yixing, Jiangsu	1	02/2001
Yixing, Jiangsu	1	04/2007
Dongtai, Jiangsu	3	04/2007
Gaoyou, Jiangsu	1	04/2007
Haian, Jiangsu	22	04/2007
Suizhou, Hubei	43	09/2007
Tongzhou, Jiangsu	19	05/2007
Chaohu, Anhui	2	05/2008
Hefei, Anhui	4	05/2008
Hangzhou, Zhejiang	10	05/2008
Liu'an, Anhui	3	05/2008
Wuhu, Anhui	3	05/2008

2.2. Determination of sensitivity of S. sclerotiorum to iprodione and bosaclid

Technical iprodione (96.5% a.i., Heyi Agricultural Chemical Co. Ltd., Zhejiang, China) was dissolved in acetone at a concentration of 100 mg/ml, and added to PDA medium after sterilization to produce final concentrations of 0, 0.1, 0.2, 0.4, and 0.8 μg/ml. To determine sensitivity of *S. sclerotiorum* to iprodione, a 5-mm mycelial plug was taken from the edge of a 2-day-old colony and placed onto the center of PDA plates amended with iprodione at each of the above concentrations. For each isolate, two replicates per concentration were used. The radial growth (colony diameter) of each isolate was measured with the original mycelial plug diameter (5 mm) subtracted from this measurement, after 2 days of incubation at 25 °C in the dark. For each plate, the average colony diameter measured in two perpendicular directions was used for calculation of the 50% effective concentration (EC_{50}), which is the fungicide concentration that results in 50% mycelial growth inhibition. EC_{50} values were calculated with a computer program DPS (Data Processing System) (Hangzhou Reifeng Information Technology Ltd., Hangzhou, China). The experiment was performed twice. Because the two experiments did not differ significantly ($P > 0.05$), the average EC_{50} values from the two experiments for each isolate were used in the data analysis.

To determine sensitivity of *S. sclerotiorum* to boscalid, boscalid (50% a.i., BASF Agricultural Chemical, Germany) was dissolved in sterilized water, adjusted to a concentration of 100 mg/ml, and added to YBS medium (10 g yeast extract, 10 g Bacto peptone, and 20 g sodium acetate in 1 L deionized water) to produce final concentrations of 0, 0.01, 0.03, 0.1, 0.3, and 1.0 μg/ml. Determination of sensitivity of *S. sclerotiorum* to this compound was conducted using a previously published protocol [11]. Briefly, 2 ml of fungicide-amended YBS was added to each well of 24-well tissue culture plates with 16.2 mm well diameter. Each well was then inoculated with a 5-mm mycelial plug taken from the edge of a 2-day-old culture. Three replicate wells were used for each isolate at each fungicide concentration. The culture plates were put into plastic bags to reduce evaporation. After incubation at 25 °C in darkness for 7 days, colony diameter in each plate was measured in two perpendicular directions with the original mycelial plug diameter (5 mm) subtracted from each measurement. The EC_{50} was calculated for each isolate as described above. The experiment was repeated twice. The average EC_{50} values from two experiments for each isolate were used in the data analysis since the two experiments did not differ significantly ($P > 0.05$).

2.3. Induction of iprodione-resistant (IR) isolates in vitro

Three iprodione-sensitive (IS) isolates HA17, SZ31, and SZ45 were randomly selected and used for induction of IR isolates by growing on PDA medium amended with iprodione at 10 and 50 μg/ml in the 1st and 2nd generation, respectively. For the first generation, after plates were incubated at 25 °C for 2 weeks, a growing (resistant) sector developed in the plate. A mycelial tip from the sector was transferred to a PDA plate amended with iprodione at 50 μg/ml. After two generations, three IR isolates, HA17-IR, SZ31-IR, and SZ45-IR were obtained from the wild-type isolates HA17, SZ31, and SZ45, respectively. To determine the stability of IR isolates, each IR isolate was grown on PDA without any fungicide for 3 days, and then transferred to a fresh PDA plate for 10 generations. The EC_{50} values to iprodione of the IR isolates at 1st, 5th, and 10th generation were determined using the same