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Sensitivity of *Sclerotinia sclerotiorum* to fludioxonil: *In vitro* determination of baseline sensitivity and resistance risk

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ABSTRACT

This work determined the sensitivity of field populations of *Sclerotinia sclerotiorum* (Lib.) de Bary before exposure to the fungicide fludioxonil (= baseline sensitivity) and assessed the risk of fludioxonil resistance. The mean EC_{50} (Effective Concentration) and Minimum inhibitory concentration (MIC) values for fludioxonil based on inhibition of mycelial growth of 120 wild-type isolates were 0.015 ± 0.005 μg/ml and <0.05 μg/ml, respectively. Positive cross-resistance was not detected between fludioxonil and benzimidazole fungicides but was detected between fludioxonil and dicarboximide fungicides which are considered as high resistance risk fungicides by FRAC, even though these fungicides have different molecular structures. By growing wild-type isolates on potato dextrose agar (PDA) containing sublethal concentrations of the fungicide, we obtained four fludioxonil-resistant mutants with resistance factors (EC_{50} resistant/EC_{50} sensitive phenotypes) >2000. The laboratory fludioxonil mutants were less fitter than their parental isolates in terms of mycelial radial growth, pathogenicity and sclerotial production. Moreover, on PDA amended with NaCl, the laboratory fludioxonil mutants grew more slowly than their fludioxonil-sensitive parents, especially at lower concentrations of NaCl. According to the fitness of mutants and the cross-resistance between fludioxonil and dicarboximide fungicides, phenylpyrroles can be considered to pose a moderate resistance risk. In a field trial, fludioxonil provided greater control (over 90% disease control) of *S. sclerotiorum* than iprodione.

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a cosmopolitan fungal pathogen that attacks more than 370 species of higher plants (Boland and Hall, 1994), such as soybean, common bean, sunflower, canola, and oilseed rape. Infection can occur on leaves, stems and pods at different crop developmental stages (Abawi and Grogan, 1979; Sylvester-Bradley and Donald, 1984; Dai et al., 2006). The disease caused by *S. sclerotiorum* is important in many countries, including Canada (Bardin and Huang, 2001), the United States (Purdy, 1979; Bolton and Nelson, 2006), Australia (Letham et al., 1976), and China (Zhou and Luo, 1994). In China, where oilseed rape is the major oilseed crop with approximately 70 × 10^6 ha in production (Zhao and Meng, 2003), Sclerotinia stem rot reduces yield from 10 to 80% and reduces oil quality (Anonymous, 1975).

During the 1980s, a benzimidazole fungicide, carbendazim (MBC), was applied for control of Sclerotinia stem rot in China. MBC subsequently failed to control the pathogen, however, because of the emergence of MBC-resistant populations of *S. sclerotiorum* in the field (Pan, 1998; Shi et al., 2000; Li et al., 2003; Zhang et al., 2003). With the recognition that MBC was often ineffective, growers then used dicarboximide fungicides (e.g., dimethachlon, iprodione, procymidone and vinclozolin) to control plant diseases incited by *S. sclerotiorum*, *Sclerotinia homoeocarpa* F. T. Bannett, and *Sclerotinia minor* Jagger for many years (Brenneman et al., 1987; Smith et al., 1991, 1995; Hubbard et al., 1997; Mueller et al., 2002; Matheron and Porchas, 2004; Jo et al., 2006). Although the mode of action of dicarboximide fungicides and the resistance mechanism of plant pathogens to these compounds are not well understood, it appears that repeated applications of dicarboximide fungicides also select for resistant strains of *S. sclerotiorum* (Ma et al., 2009), *Sclerotinia homoeocarpa* (Jo et al., 2006), *Sclerotinia minor* (Smith et al., 1995), *Botrytis cinerea* Pers. (Katan, 1982; Beever and Brien, 1983; Leroux et al., 2002), and *Alternaria* sp. (Ma and Michailides, 2004). Thus, it is necessary to find more effective fungicides to replace the compounds that are currently

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used to control Sclerotinia stem rot (Brent, 1988) and other fungal diseases.

Fludioxonil [4-(2, 2-difluoro-1, 3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile] is a phenylpyrrole, a novel class of non-systemic fungicide (Gehmann et al., 1990). The phenylpyrrole fungicides are derivates of the antibiotic pyrrolnitrin, which is produced by various *Pseudomonas* species (Errampalli, 2004). According to some previous studies, fludioxonil strongly interferes with mycelial growth and conidial germination of *Penicillium expansum* Link (Errampalli, 2004) and *Botrytis cinerea* (Leroux, 1996). Field resistance to fludioxonil in *Alternaria* spp. from crucifers and *Penicillium digitatum* (Pers.) Sacc. from lemon has been also reported (Iacomi-Vasilescu et al., 2004; Kanetis et al., 2006). Although fludioxonil has been widely used throughout the world, its application for control of Sclerotinia stem rot has not been reported.

The objectives of the present study were to (i) establish the baseline sensitivity (sensitivity before exposure to the fungicide) to fludioxonil in *S. sclerotiorum* populations from different oilseed rape fields in Jiangsu Province, China; (ii) recover laboratory-resistant mutants of *S. sclerotiorum* to fludioxonil and investigate resistance stability; (iii) evaluate the risk of *S. sclerotiorum* developing resistance to fludioxonil by comparing the biological properties of fludioxonil-sensitive and fludioxonil-resistant strains and exploring the cross-resistance patterns between fludioxonil and other well-known fungicides, such as benzimidazoles and dicarboximide; and (iv) test the efficacy of fludioxonil in controlling Sclerotinia stem rot in a field in Jiangsu Province where MBC was extensively used for decades but no longer controls the disease.

2. Materials and methods

2.1. Fungicides and media

Technical grade dimethachlon (90.0%), MBC (95.0%), fludioxonil (97.9%), and iprodione (96.2%) were kindly provided by the Wenzhou Pesticide Factory; Shenyang Research Institute of Chemical Industry; Agrolex P. Ltd., Syngenta (China); and Jiangsu kuaida Agrochemical Co. Ltd., respectively. They were dissolved in methanol, hydrochloric acid (0.1 mol/L), methanol, and acetone, respectively, to obtain 10 mg of active ingredient/ml for the stock solution. The stock solutions were added to cooled (42–50 °C) but non-solidified autoclaved media, and the pH was adjusted to 6.8 in all media using 0.1 M HCl. Commercially produced fludioxonil (a 2.5% SC, a formulation used for coating of seeds), MBC (50% WP), iprodione (50% WP), and dimethachlon (46% WG) were purchased in Nanjing (Jiangsu Province, China). The SC formulation of fludioxonil was used because a spray formulation is not commercially available.

Potato dextrose agar (PDA) was prepared with 200 g potato, 20 g agar, and 20 g dextrose per liter of distilled water. Potato dextrose broth (PDB) was prepared with 200 g potato and 20 g dextrose per liter of distilled water.

2.2. Collection of isolates

Isolates ($N = 120$) of *S. sclerotiorum* used in the study were collected from thirteen oilseed rape fields in Jiangsu Province of China from 2007 to 2008. In each field, several plants with typical 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 oilseed rape plants. The sclerotia were surface disinfested 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. 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 cultures were maintained on PDA slants at 4 °C.

2.3. In vitro baseline sensitivity to fludioxonil

The effect of fludioxonil on hyphal growth (Zhou et al., 1994) was determined *in vitro* by transferring plugs (5 mm in diameter) of mycelium from the leading edge of an actively growing colony to a series of PDA plates containing 0.00625, 0.0125, 0.025, 0.05 or 0.1 μg/ml fludioxonil. Plates without fludioxonil were used as a control. Each isolate was incubated at 25 °C for 2 days with three replicates. Mean colony diameter (minus the diameter of the inoculation plug) was measured for each treatment and expressed as a percentage of growth inhibition. The median effective fludioxonil concentration (EC_{50}) for each isolate was calculated by regressing percentage growth inhibition against the log of fungicide concentration (Zhou et al., 1994). The experiment was performed twice.

2.4. In vitro selection for fludioxonil resistance, test the stability and cross-resistance of fludioxonil-resistant mutants

Fresh mycelial plugs (5 mm in diameter) from colony margins of 100 randomly selected sensitive isolates were transferred to PDA plates containing 0.05 μg/ml (Minimum inhibitory concentration, MIC) fludioxonil and incubated in the growth chamber (25 °C and 12 h photoperiod) for 3 days. Any fast-growing sectors from the otherwise restricted colonies were selected and transferred to PDA plates containing 30 μg/ml fludioxonil. After 30 days at 25 °C, those colonies that continued to grow on the PDA containing 30 μg/ml fludioxonil were identified as fludioxonil-resistant phenotypes. The EC_{50} of resistant isolates was determined by assessting the inhibition of hyphal growth (Zhou et al., 1994). The level of fungicide resistance, termed the resistance factor (RF), was calculated as the EC_{50} of the resistance phenotype divided by the EC_{50} of the sensitive parental isolate.

The laboratory-induced fludioxonil-resistant mutants were transferred eight times onto new fungicide-free PDA plates and PDA containing 30 μg/ml fludioxonil with three plates per isolate. The fungi were allowed to grow for 2 days at 25 °C between each transfer. The EC_{50} values were then determined again as described earlier to determine whether resistance was stable. In addition, each of the mutant phenotypes was stored on fungicide-free PDA slants with three slants per isolate at 4 °C for 60 days to determine whether resistance was stable. The experiments were performed twice.

After resistant mutants and their sensitive parental isolates were subcultured on PDA for 2 days at 25 °C in a growth chamber, 5-mm-diameter mycelial agar plugs were transferred from the margins of the colonies onto PDA containing a series of concentrations of dimethachlon, carbendazim, iprodione, or fludioxonil in 9-cm Petri dishes (Table 1). There were three replicate plates per concentration,

Table 1
Concentrations of fungicides used to determine cross-resistance of fludioxonil-resistant mutants of *Sclerotinia sclerotiorum*.

Fungicides	Fungicide concentrations (μg/ml)	
	Fludioxonil–sensitive isolates[a]	Fludioxonil–resistant isolates[a]
Dimethachlon	2, 1, 0.5, 0.25, 0.13,	300, 200, 150, 75, 37.5, 18.75,
Iprodione	0.4, 0.3, 0.2, 0.15, 0.1, 0.08,	300, 150, 75, 37.5, 18.75,
Fludioxonil	0.1, 0.05, 0.03, 0.01, 0.06, 0.03,	100, 50, 25, 12.5, 6.25,
Carbendazim	0.9, 0.5, 0.25, 0.13, 0.06, 0.03,	0.9, 0.5, 0.25, 0.13, 0.06, 0.03,

[a] Fludioxonil-sensitive isolates were field isolates and fludioxonil-resistant mutants were induced in the laboratory.