Pesticide Biochemistry and Physiology 106 (2013) 61–67

Contents lists available at SciVerse ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Effect of phenylpyrrole fungicide fludioxonil on morphological and physiological characteristics of *Sclerotinia sclerotiorum*

Yabing Duan [1], Changyan Ge [1], Shengming Liu, Changjun Chen, Mingguo Zhou *

College of Plant Protection, Nanjing Agricultural University, Key Laboratory of Monitoring and Management of Crop Diseases and Pest Insects, Ministry of Agriculture, Key Laboratory of Pesticide, Jiangsu Province, Nanjing 210095, China

ARTICLE INFO

Article history:
Available online 19 April 2013

Keywords:
Sclerotinia sclerotiorum
Fludioxonil
Mycelia morphology
Biological characterization

ABSTRACT

In this study, the effect of fludioxonil on morphological and physiological characteristics of *Sclerotinia sclerotiorum* has been investigated. The results indicated that fludioxonil had a strong inhibition on mycelia growth of *S. sclerotiorum*. After fludioxonil treatment, cell membrane permeability, glycerol content, POD and PAL activity increased markedly, but oxalate and EPS content significantly decreased. The protective and curative test of fludioxonil suggested that protective effect was better than curative either on leaves or on stems of oilseed rape. These results will increase our understanding of action mechanism of fludioxonil against *S. sclerotiorum*.

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is an aggressive phytopathogen with a broad host range and a worldwide distribution. At least 408 species of plants from 278 genera in 75 families are susceptible to this pathogen, including numerous important crops and weeds [1–4]. The pathogen can cause serious losses of yield and quality for a number of important field and vegetable crops in many countries [1,3,5–11]. Since diseases caused by *S. sclerotiorum* have traditionally been difficult to control, the search for host resistance has been an important objective of research programmes on this pathogen [12]. However, levels of resistance that can be relied upon as a primary control measure have been difficult to achieve. At practice, application of fungicides is the principal tool for controlling Sclerotinia diseases on most crops [5,13]. Unfortunately, as a result of the continuous use of fungicides at high concentrations in recent years, the risk of development of resistance of *S. sclerotiorum* to fungicides has increased in various countries and leads to control failures [14–20].

Fludioxonil (trade name Celest in China) is a phenylpyrrole, a novel class of non-systemic and broad-spectrum fungicide [21]. The phenylpyrrole fungicides are derivates of the antibiotic pyrrolnitrin, which is produced by various *Pseudomonas* species [22]. According to some previous studies, fludioxonil strongly interferes with mycelia growth and conidial germination of *Penicillium expansum* Link [22] and *Botrytis cinerea* [23]. Fludioxonil has been used extensively as seed treatment for the control of various dieases for many years [24–28]. But fludioxonil has currently been developed as new spray formulations for controlling some diseases [29–31]. In our previous study, the baseline sensitivity of *S. sclerotiorum* to fludioxonil was established and fludioxonil has high fungicidal activity against *S. sclerotiorum in vitro* and has been used for control of oilseed rape stem rot in field [32]. However, presently, fludioxonil is not registered for controlling oilseed rape stem rot in China. Meanwhile, action mechanism of fludioxonil against *S. sclerotiorum* is unclear. Therefore, the objective of this study was to determine the effect of fludioxonil on the morphological and physiological characteristics of *S. sclerotiorum*. This will increase our understanding of action mechanism of fludioxonil against *S. sclerotiorum* and other phytopathogen and could provide new reference data for the management of Sclerotinia stem rot caused by *S. sclerotiorum*.

2. Materials and methods

2.1. Fungicides, strains and culture conditions

Technical-grade fludioxonil (97.9%) was provided by Syngenta Biotechnology (China), Co. Ltd. The fungicide was dissolved in methanol at 10 mg/mL and kept as a stock solution.

S. sclerotiorum isolates HA61, NT18, SZ45 used in this study were collected from different geographic oilseed rape fields in Jiangsu Province of China. These isolates were generated from a single sclerotium and maintained on PSA slants at 4 °C.

Potato sucrose agar (PSA) was prepared with 200 g of potato, 20 g of agar, and 20 g of sucrose per liter of distilled water. Potato sucrose broth (PSB) had the same contents as PSA but lacked agar.

* Corresponding author. Fax: +86 025 84395641.
E-mail addresses: yabing0324@hotmail.com (Y. Duan), mingguo.zhou@yahoo.com.cn (M. Zhou).
[1] Joint first authorship.

0048-3575/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pestbp.2013.04.004

2.2. Sensitivity to fludioxonil

Three *S. sclerotiorum* isolates used in this study were tested for fludioxonil sensitivity. Mycelia plugs (5 mm in diameter) from the leading edge of an actively growing colony were transferred to a series of PSA plates containing 0, 0.00625, 0.0125, 0.025, 0.05, or 0.1 μg/mL fludioxonil. 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 based on linear regression of colony diameter on log-transformed fungicide concentration [33]. The experiment was performed twice.

2.3. Effect of fludioxonil on mycelial morphology of S. sclerotiorum

Mycelia plugs from the edge of a 2-day-old colony were transferred to PSA plates containing 0.025 μg/mL fludioxonil. Plates without fludioxonil were used as the control. After 2 days at 25 °C, the medium area (10 mm × 10 mm) of hyphal tips was cut and put on slide glass. The morphological change of mycelia was observed by light microscope. Each isolate was determined with three replicates. The experiment was performed twice.

2.4. Effect of fludioxonil on cell membrane permeability of S. sclerotiorum

For each of three *S. sclerotiorum* isolates, mycelial plugs (5 mm diameter) from the margins of 2-day-old colonies on PSA were placed in 250-mL flasks (5 plugs per flask) containing 100 mL of PSB. The flasks were placed on a rotary shaker (175 rpm at 25 °C). After 36 h, partial flasks were amended with fludioxonil at the ultimate concentration 0.025 μg/mL. The flasks were shaken for an additional 36 h, mycelia were collected on double gauze and washed twice with double-distilled water. After filtration in vacuum for 20 min, 0.5 g of mycelia per sample was suspended in 20 mL of double-distilled water. After 0, 5, 10, 20, 40, 60, 80, 100, 120, 140, 160, and 180 min, the electrical conductivity of the double-distilled water was measured with a conductivity meter (CON510 Eutech/Oakton, Singapore) to assess the extent of leaching of cell contents through cell membranes. After 180 min, the mycelia were boiled for 5 min, and final conductivity was measured. The relative conductivity of mycelia was calculated as:

$$\text{Relative conductivity } (\%) = \text{Conductivity}/\text{Final conductivity} \times 100.$$

All tests had four replicates, and each experiment was repeated twice.

2.5. Glycerol content of mycelia

Glycerol in mycelia was measured by the cupric glycerinate colorimetry method [34] with some modification. For preparation of a standard curve for glycerol, known concentrations of glycerol were prepared with double-distilled water. 1 mL volume of a $CuSO_4$ solution (0.05 g/L) and 3.5 mL of a NaOH solution (0.05 g/mL) were mixed and added to 50 mL flasks containing 10 mL of glycerol at known concentrations (0, 0.0025, 0.003, 0.004, 0.005, 0.006, 0.008, and 0.01 g/mL). The flasks were placed on a rotary shaker (100 rpm) for 12 min, and the liquid was filtered through lens paper. The concentration of cupric glycerinate was measured at 630 nm, which is the maximum absorbance (A_{max}) frequency of cupric glycerinate, with a spectrophotometer. In this case, the greater the absorbance, the higher the glycerol concentration. A standard curve was generated by plotting absorbance against glycerol concentration.

For determination of glycerol in mycelia of each of three *S. sclerotiorum* isolates, mycelia were prepared as described section 2.4. Mycelia (0.5 g) per sample were ground with a freezing pestle and mortar containing 20 mL of sterile-distilled water and quartz sand. The suspensions were transferred to 50-mL centrifuge tubes and heated at 80 °C for 15 min. Then they were centrifuged at 8500 rpm for 10 min. The glycerol content of the supernatants was determined with the standard curve. Sterile-distilled water was used as the control. There were four replications for each treatment, and the test was repeated.

2.6. Exopolysaccharide (EPS) content

The quantity of EPS produced by each of three *S. sclerotiorum* isolates was determined by a modified version of the phenol–sulfuric acid method [35,36]. For preparation of an EPS standard curve, 2 mL of a glucose solution (0, 20, 40, 60, 80, 100, 120, 160, and 200 μg of glucose/mL of double-distilled water) and 1 mL of a 5% phenol solution were added to test tubes, which were mixed with a vortex mixer. A 5-mL volume of concentrated H_2SO_4 was then added either directly to the liquid surface in 2 or 10 s, or slowly down the side of the test tube. The test tubes were then closed with rubber plugs, mixed with a vortex mixer for 10 s, and incubated for 30 min at 25 °C. Absorbance of the solution was measured at 490 nm. In this case, the greater the absorbance, the higher the glucose concentration. A standard curve was generated by plotting absorbance against glucose concentration.

For determination of EPS content, mycelial plugs (5 mm diameter) from the margins of 2-day-old colonies on PSA were placed in 250-mL flasks (five plugs per flask) containing 100 mL of PSB. The flasks were placed on a rotary shaker (175 rpm at 25 °C). After 36 h, partial flasks were amended with fludioxonil at the ultimate concentration 0.025 μg/mL. The flasks were shaken for an additional 36 h. The flask contents were centrifuged at 10000 rpm for 20 min, and the supernatants were collected. EPS was precipitated from 1 mL of each supernatant with three volumes of absolute ethanol and then dried. The EPS were dissolved in 8 mL of distilled water and quantified with the standard curve. Sterile-distilled water was used as a control. There were four replications for each treatment, and the test was repeated.

2.7. Oxalic acid content

Iron (III) and oxalate form a complex that discolors the complex of iron (III) and sulfosalicylic acidic under acidic conditions [37]. The content of oxalate was measured indirectly by measuring the intensity of the colour of the complex of iron (III) and sulfosalicylic acidic at 510 nm with a spectrophotometer. For preparation of a standard curve for oxalate, 2 mL of $FeCl_3$ solution (0.5 mg/mL), 20 mL of HCl–KCl buffer solution (KCl 50 mM, pH = 2), and 1.2 mL of sulfosalicylic acid solution (5 mg/mL) were added to a 50-mL flask. Different volumes (0, 0.1, 0.2, 0.4, or 0.8 mL) of sodium oxalate solution (2 mg/mL) were then added, and double-distilled water was used to increase the volume to 25 mL. The flasks were vortex-stirred for 10 s and incubated for 30 min at 25 °C. Double-distilled water was used as a blank. Absorbance was measured at 510 nm with a spectrophotometer. In this case, the greater the absorbance, the lower the sodium oxalate concentration. A standard curve was generated by plotting absorbance against sodium oxalate concentration.

For determination of oxalic acid content, mycelial plugs (5 mm diameter) from the margins of 2-day-old colonies on PSA were placed in 250-mL flasks (five plugs per flask) containing 100 mL of PSB amended with fludioxonil at the ultimate concentration