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Effects of a novel SDHI fungicide pyraziflumid on the biology of the plant pathogenic fungi *Bipolaris maydis*

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ABSTRACT

Pyraziflumid is a novel member of succinate dehydrogenase inhibitor (SDHI) fungicide. Southern corn leaf blight (SCLB) caused by *Bipolaris maydis* is an important foliar disease of maize crop. In this study, baseline sensitivity of *B. maydis* to pyraziflumid was determined using 100 strains of *B. maydis* collected from different geographical regions in Jiangsu Province of China during 2015 and 2016, and EC_{50} values ranged from 0.0309 to 0.8856 μg/ml with the average value of 0.2780 ± 0.2012 μg/ml for mycelial growth, and 0.032 to 0.9592 μg/ml with the average value of 0.3492 ± 0.2450 μg/ml for conidium germination. After treatment with pyraziflumid, the distribution of cell nucleus and septum of mycelium was not changed, but hyphae of offshoot and conidia production decreased, cell secretion decreased, the cell membrane was damaged, mycelium electrolyte leakage increased, and organelles in mycelial cell dissolved and vacuolated. The protective and curative activity test of pyraziflumid suggested that pyraziflumid had great control efficiency against *B. maydis* on detached corn leaves. In protective activity assay with application of pyraziflumid at 5 μg/ml and 10 μg/ml, the control efficacy reached to 87.32% and 100% respectively. In curative activity assay with application of pyraziflumid at 20 μg/ml and 50 μg/ml, the control efficacy reached to 82.10% and 100% respectively.

1. Introduction

Maize is a widely cultivated crop that provides food, animal feed, and a source of biofuel [1–4]. Although maize has been growing for thousands of years, disease remains a worldwide concern that causes the loss of production and the decline of quality [5–7]. Southern corn leaf blight (SCLB) caused by filamentous fungus *Bipolaris maydis* is an important foliar disease of maize crop and often occurs in warm and humid areas [8–11]. In 1970, the SCLB epidemics sharply dropped the corn and its yield by 50% in the United States, causing catastrophic losseds to the country's economy [12–15]. The disease is also a primary concern mostly in the Yellow-Huai-Hai River plain regions of China [16,17]. Planting resistant cultivars and application of fungicides are the major methods for controlling SCLB [8]. Despite the use of resistant cultivars and hybird varieties, SCLB is still a threat to maize production due to racial variations [18–21]. In China, amobam, the mixture of azoxystrobin and propiconazole, and the mixture of azoxystrobin and tebuconazole were registered for controlling SCLB (http://www.icama.org.cn/hysj/index.jhtml). The fungicides for controlling the disease are limited. So it is necessary to screen candidate fungicides.

Usually, the succinate dehydrogenase inhibitor (SDHI) fungicides have a broad spectrum of activity against many phytopathogens because SDHI fungicides interfere the respiration of plant pathogens by inhibiting the activity of complex II enzymes [22–25]. The fungicide pyraziflumid (development code number: NNF-0721), N-(3′,4′-difluoro [1,1′-biphenyl]-2-yl)-3-(trifluoromethyl)pyrazine-2-carboxamide, developed by Nihon Nohyaku CO., LTD. is a novel member of SDHI fungicides [26–28]. Its molecular formula is $C_{18}H_{10}F_5N_3O$ and its structural formula is shown in Fig. 1.

Pyraziflumid has not been registered for controlling SCLB in China. In the screening experiment, we found that pyraziflumid had strong inhibitory effect on mycelial growth of *B. maydis in vitro*, which indicated that pyraziflumid may be an potential fungicide for controlling SCLB. The effects of pyraziflumid on *B. maydis* has not been reported. So the objectives of this study were (i) determine sensitivity distribution of *B. maydis* populations to the novel fungicide pyraziflumid by establishing baseline sensitivities (sensitivity before exposure to the fungicide) of 100 *B. maydis* isolates collected from various regions of Jiangsu Province of China to pyraziflumid by mycelial growth assay and conidium germination assay respectively. (ii) understand the effects of pyraziflumid on physiological characteristics of *B. maydis*. (iii) analyze the protective and curative activity of pyraziflumid against *B. maydis* on detached leaves of maize. This could provide new reference data for the management of SCLB caused by *B. maydis* and will increase our

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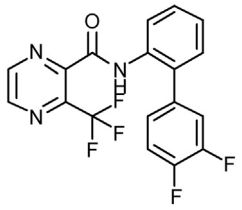

Fig. 1. The chemical structure of pyraziflumid.

understanding the mode of action of pyraziflumid against *B. maydis* and other phytopathogens.

2. Materials and methods

2.1. Collection of B. maydis isolates

Maize plants with typical symptoms of *B. maydis* infection were collected from Huaian, Lianyungang and Xuzhou of Jiangsu Province of China between 2015 and 2016. Maize leaves with lesions were cut into pieces (5 × 5 mm), disinfected in 1% NaClO for 3 min, rinsed three times with sterile water, and placed on potato dextrose agar (PDA) medium plates amended with 100 μg/ml streptomycin sulfate [29]. After 4 days of incubation at 25 °C, *B. maydis* was isolated from the edge of the colony and then transferred to a fresh PDA plate. In total, 100 isolates were obtained. All of the isolates were maintained on PDA slants and stored at 4 °C.

2.2. Fungicides and culture media

Technical grade pyraziflumid (active ingredient 94.8%; Nihon Nohyaku CO., LTD) was dissolved in methanol at 1×10^4 μg/ml and kept as a stock solution. PDA medium was made from 200 g potato, 20 g dextrose and 18 g agar per liter of distilled water and used for regular growth of *B. maydis*. Water agar (WA) was prepared with 16 g agar per liter of distilled water and used for conidium germination. YEPD liquid medium was made from 0.3% (w/v) yeast extract, 1% peptone, 2% glucose per liter of distilled water.

2.3. Baseline sensitivity of mycelial growth of B. maydis populations to pyraziflumid

The baseline sensitivity of mycelial growth of *B. maydis* populations to pyraziflumid was determined by analyzing the distribution of EC_{50} (50% effective concentration) values in 100 strains. Briefly, mycelial plugs (5 mm diameter) from the edge of 7-day-old colony of each strain were placed on a series of PDA plates containing 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1 μg/ml pyraziflumid. For each concentration, three replications were conducted. Plates without pyraziflumid were prepared as the control. The diameters (minus the diameter of the inoculation plug) of the colonies were measured after incubation for 7 days at 25 °C in darkness. The growth inhibition as percent of control was calculated. Inhibition ratios of the fungicide to mycelial growth of *B. maydis* strains in different concentrations were calculated by the formula: (the averaged diameter of control — the averaged diameter in some fungicide concentration)/(the averaged diameter of control — 5 mm of mycelial plug). The EC_{50} values for the strains were calculated based on linear regression of colony diameter on log-transformed fungicide concentration [30,31]. The experiment was performed twice.

2.4. Baseline sensitivity of conidium germination of B. maydis populations to pyraziflumid

The baseline sensitivity of conidium germination of *B. maydis* populations to pyraziflumid was determined by analyzing the distribution of EC_{50} values in 100 strains. Briefly, the strains grew on PDA medium at 25 °C for 8 days in darkness. Conidial suspension were obtained by washing the mycelia with sterile water. Conidia were filtered with three pieces of lens wiping paper and collected by centrifuging at 5000 rpm for 5 min. The conidia were suspended with sterile water and its concentration was adjusted to 1×10^4/ml. 100 μl suspension of conidia was smeared on the surface of WA plates containing 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1 μg/ml pyraziflumid. Plates without pyraziflumid were prepared as the control. After cultured in an incubator at 25 °C for 8 h in darkness, germination was counted at three sites by the microscope (Primo Star, Zeiss, Germany) and 100 conidia were counted in each site. The standard of a conidium considered to be germinated was according to the previous study [32]. There were three replicates in the experiment and the experiment was repeated twice. Inhibition ratios of the fungicide to conidium germination of *B. maydis* strains in different concentrations were calculated by the formula: (the number of germinated conidia of control — the number of germinated conidia in some fungicide concentration)/the number of germinated conidia of control. EC_{50} value of pyraziflumid to each strain was determined according to previous study [33].

2.5. Effect of pyraziflumid on mycelial morphology of B. maydis

Mycelial plugs from the margin of a 7-day-old colony (strains YX226, YX104, YX27) were transferred to slide glass containing 1 ml PDA medium amended with 4 μg/ml pyraziflumid. Slide glass without pyraziflumid were used as the control. After 36 h of culture at 25 °C, the morphology of the top of mycelia was observed by light microscope (Olympus IX-71, Japan). There were three replications for each treatment and the experiment was repeated three times.

2.6. Effect of pyraziflumid on conidium production of B. maydis

Mycelial plugs from the margin of a 7-day-old colony (isolates YX27, YX43, YX104) were transferred to PDA plates containing 0.25, 1 or 4 μg/ml pyraziflumid. Plates without pyraziflumid were used as the control. After 8 days of culture at 25 °C in darkness, conidial suspension of each strain was obtained by washing the mycelia with 5 ml sterile water. Conidia were filtered with three pieces of lens wiping paper. Conidia concentration was counted with hemocytometer under the microscope (Primo Star, Zeiss, Germany). There were three replications for each treatment and the test was repeated three times.

2.7. Effect of pyraziflumid on nuclei and septum of B. maydis

The strain of YX43 was chosen for this test. Five mycelial plugs (5 mm in diameter) from the edge of 7-day-old colony were added into 20 ml YEPD medium in 50 ml triangular flask and the flask was shaken at 175 rpm in a rotary shaker. After the flasks were shaken at 175 rpm and 25 °C for 24 h, some flasks were supplemented with pyraziflumid at the ultimate concentration of 4 μg/ml. After the flasks were shaken for additional 12 h, hyphae were collected through three pieces of lens paper. The collected mycelia were stained with a solution containing 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml) or Calcofluor White (CFW, 83 μg/ml) for nuclei or septum distribution respectively [34,35], and observed with a fluorescence microscope (Olympus IX-71, Japan).

2.8. Effect of pyraziflumid on cell ultrastructure of B. maydis

The strain of YX43 was chosen for this test. Ten mycelial plugs (5 mm in diameter) from the edge of 7-day-old colony were added into 100 ml YEPD medium in 250 ml triangular flask and the flask was shaken at 175 rpm in a rotary shaker. After the flasks were shaken at 175 rpm and 25 °C for 60 h, some flasks were supplemented with pyraziflumid at the ultimate concentration of 4 μg/ml. After the flasks were shaken for additional 48 h, hyphae were fixed with 2.5%