1 Exploration of fungicide resistance to Fludioxonil and

Tebuconazole of Fusarium pseudograminearum, the

causal agent of wheat Fusarium crown rot

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Abstract

- 18 The damage caused by wheat soil-borne diseases, especially Fusarium crown rot (FCR), affect
- 19 the yield and quality of wheat increasingly. In year 2020, a total of 297 Fusarium
- 20 pseudograminearum strains were isolated and purified from diseased FCR wheat samples in
- 21 Hebei Province. Baseline sensitivity of *F. pseudograminearum* to Fludioxonil (0.0610±0.0367
- 22 μg/mL) and Tebuconazole (0.2328±0.0840 μg/mL) were constructed based on in vitro tests of 61
- and 82 strains, respectively. No resistance isolate to Fludioxonil was detected, while 2 low
- 24 resistance isolates to Tebuconazole were detected based on the resistance index analysis. There
- 25 was no cross-resistance between Fludioxonil and Tebuconazole. This study provides theoretical
- and practical value to monitor resistance of F. pseudograminearum to fungicides and control of
- 27 FCR.

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Introduction

- Fusarium crown rot (FCR), a soil-borne disease, causes major devastating diseases of wheat
- 31 (Triticum aestivum L.) worldwide. The occurrence of FCR has been reported in many arid and
- 32 semi-arid wheat growing continents and countries including Australia (Magee, 1957), America
- 33 (Smiley, 2005), Africa (Gargouri et al., 2011), Europe (Agustí-Brisach, 2018), the Middle East
- 34 (Hameed et al., 2012; Shikur Gebremariam et al., 2018), as well as China (Li et al., 2012; Zhang
- et al., 2015b; Xu et al., 2018). The infected wheat resulting in brown necrosis at the first two or
- 36 three internodes or the production of white blighted heads and abortive seeds in the case of a
- 37 severe attack (Scherm et al., 2013). As a consequence, significant yield losses are reported all
- 38 over the world.

- 39 Hebei province, among the wheat-corn rotation region in the North China Plain, account for
- 40 about 10% and 11.4% on the planting area and gross product, respectively. FCR has been
- 41 spreading in Hebei and leading to a potential yield loss. F. pseudograminearum was the most
- 42 commonly reported damaging Fusarium species causing crown rot of wheat, and with strong
- pathogenicity in China (Deng et al. 2020). This species has been repeatedly reported to be
- associated with Fusarium head blight (FHB) as well (Xu et al., 2015, 2018; Ji et al., 2016).
- 45 There are representative dressing agents for controlling FCR diseases in China, such as Qingxiu
- 46 (10% Difenoconazole), Cruiser (2.2% Fludioxonil + 2.2% Difenoconazole), Dividan (3%
- 47 Difenoconazole), Raxil (6% Tebuconazole), Celest (2.5% Fludioxonil), Aobairui (1.1%
- 48 Tebuconazole). The main active ingredient to fungus of these agent including Tebuconazole,
- 49 Difenoconazole and Fludioxonil. Chemical control of FCR exhibits the most effective method to
- 50 limit disease, but repeated fungicidal applications reduced sensitivity to strains of *Fusarium* spp.,
- when fungicides were employed, thus increase the risk of severe plant disease and bring a series
- of problems such as environmental pollution and residual toxicity. Determination of the
- susceptibility of Fusarium pathogen to fungicides in wheat most focused on the prevalent F.
- 54 graminearum causing Fusarium head blight (FHB) (de Chaves et al. 2022; Breunig and Chilvers,
- 55 2021). Yin et al (2021) showed that Carbendazim had a strong inhibitory effect on F.
- 56 pseudograminerum population from Henan, China, with baseline sensitivity of (0.755±0.336)
- 57 μg/mL. Little information is available about the activity and risk of resistance to Fludioxonil and
- Tebuconazole in F. pseudograminearum. Consequently, this study aimed to evaluate the
- 59 susceptibility and cross-resistance of F. pseudograminearum from field populations to
- 60 Fludioxonil and Tebuconazole. The results will provide reference for the resistance monitoring
- of FCR predominant pathogen and the rational application of Trizole and Fludioxonil in the
- 62 control of wheat crown rot in Hebei, China and/or all over the world.

Materials & Methods

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F. pseudograminearum isolates collection

- In late April to late May of 2020, when wheat stem exhibiting remarkable crown rot symptoms,
- 66 FCR diseased samples were collected at different wheat grown regions in Hebei Province, China
- 67 (Table 1). The infected stalks were sampled randomly, with each region at least 30 isolates, and
- 68 geographical distance at least 10 km. A total of 297 field isolates of F. pseudograminearum was
- 69 isolated according to Deng et al. (2020). The single-spore isolation was performed and pure
- strains were re-cultured on the PDA medium.

Fungicide-containing medium preparation

- 72 Technical grade Fludioxonil (98% active ingredient [a.i.]) and Tebuconazole (97% a.i.) -applied
- 73 for the in vitro sensitivity assay were kindly supplied by Hebei Weiyuan Biochemical Co. Ltd.
- 74 Stock solutions of Fludioxonil was obtained by dissolving it in methyl alcohol to yield a
- concentration of 1000 mg/mL. Tebuconazole was dissolved in acetone. PDA plates was amended
- 76 with Fludioxonil to give serially final concentrations of 0.015, 0.03, 0.06, 0.12, 0.24 and 0.48 μg
- a.i./mL, Tebuconazole with concentration of 0.025, 0.1, 0.4, 1.6, 6.4 µg a.i./mL, PDA plates
- amended with 0.1% (v/v) methyl alcohol or acetone were served as control.

79 Baseline sensitivity of F. pseudograminearum to Fludioxonil and Tebuconazole

- 80 For sensitivity test, at least 7 isolates from each wheat geographic region were randomly selected
- 81 to form a subset poapulation. 61 F. pseudograminearum isolates were assessed to Fludioxonil
- and 82 isolates to Tebuconazole by mycelial growth rate method (Secor and Rivera, 2012). In
- short, 0.7 cm mycelial plugs from the edge of pre-cultured colonies were transferred upside
- down onto the center of PDA plates amended with Fludioxonil. Mean radial mycelial growth
- was measured for each treatment by criss-cross after 3-4 days of incubation at 27°C in the dark.
- 86 Effective concentration for 50% growth inhibition (EC₅₀) was calculated using the fungicide
- 87 concentrations and the corresponding inhibition rate of mycelial growth.
- Colony diameter (cm) = measured colony diameter-fungal plug diameter (0.7 cm)
- Relative inhibition (%) = [(colony diameter of control colony diameter of treatment)/colony diameter of control] × 100
- Fungicide concentration ($\mu g/mL$) converted into a base-10 logarithmic value (x) and inhibition
- of mycelial growth subjected to the Statistical Package of the Social Science (SPSS21.0)
- 93 software to make a linear regression line to obtain the virulence regression equation (y = a + bx)
- and the correlation coefficient (r) (Stein and Kirk, 2003).
- The baseline sensitivity was established using the frequency distribution of EC₅₀ values (Hu et al. 2020).

Fungicides resistant strains and their occurrence frequency

- The fungicides resistance index of each strain was assessed by the formula below, and then F.
- 99 pseudogramineum to Fludioxonil and Tebuconazole can be divided according to the following
- 100 criteria, sample classified LR, MR and HR were all taken as fungicide resistant strains.
- Resistance index (RI) = EC_{50} of the tested isolate/ baseline sensitivity
- Sensitive strain (S): $0 < RI \le 5$, Low resistance strain (LR): $5 < RI \le 10$, Middle resistance strain
- 103 (MR): $10 < RI \le 40$, High resistance strain (HR): 40 < RI.
- Frequency of resistant strains (%) = (resistant strains/tested whole strains) \times 100
- 105 Cross-resistance analysis
- 106 A subset of 54 F. pseudograminearum isolates were used to assess their cross resistance. The
- 107 linear regression analysis was carried out by using EC₅₀ of Fludioxonil to strain as X-axis and
- 108 EC₅₀ of Tebuconazole to strain as Y-axis, the linear regression equation y = bx + a was
- 109 constructed. According to the determination coefficient (R²), the significance level of
- independent sample T test (P value) and Pearson correlation analysis, cross resistance between
- 111 Fludioxonil and Tebuconazole were analyzed.
- 112 Data analysis

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- 113 The SPSS version 21 and Microsoft Office Excel 2010 program package were used for statistical
- analysis. The in vitro experimental design was completely randomized consisting of three
- replications for each treatment and were repeated twice. Average of results were calculated for
- no significant difference (P>0.05) was observed in mycelium growth among the two
- experiments. Means comparison of the treatments was performed by LSD test ($P \le 0.05$).

- Pearson correlation analysis was carried out by SPSS 21., and Duncan's new complex range
- method was used to test the significance of differences.

120 Results

- 121 Sensitivity of mycelial growth of *F. pseudograminearum* to Fludioxonil
- 122 EC₅₀ values for all the 61 isolates were combined to establish sensitivity baseline. The EC₅₀
- values of the corresponding isolates for mycelial growth assays were continuous, range from
- 124 0.0165 to 0.1789 µg/mL, with mean value of (0.0610 ± 0.0367) µg/mL (Fig. 1A). The variation
- factor (the ratio of the maximum to the minimum EC_{50} values) was 10.84. Based on the EC_{50}
- value of the tested strains, the frequency distribution showed a unimodal curve with a positive
- skew (Fig. 1B). The strains with EC₅₀ values in the range of $0.03-0.06 \mu g/mL$ showed the highest
- 128 frequency (55.74%). The average EC₅₀ value of 0.0610 μg/mL was preliminarily determined as
- the baseline sensitivity for Fludioxonil of *F. pseudograminearum*. No resistance isolate of *F.*
- 130 pseudograminearum was observed in the field subset papulation from those we tested.
- The mean EC_{50} values of F. pseudograminearum strains collected from different geographic
- regions were with significant different (Table 1). The strains with the most sensitivity (<0.03
- 133 µg/mL) were form from Shijiazhuang, Baoding and Cangzhou of Hebei, China. The strains with
- the highest EC₅₀ were from Shijiazhuang and Handan. Strains from Baoding showed the lowest
- sensitivity variation on Fludioxonil, while strains from Shijiazhuang showed the highest.
- 136 Sensitivity of mycelial growth of *F. pseudograminearum* to Tebuconazole
- The EC₅₀ values of 82 isolates for mycelial growth assays to Tebuconazole were continuous,
- range from 0.0417 to 1.5072 μ g/mL. The variation factor was 50.21. Based on the EC₅₀ value of
- the tested strains, the frequency distribution showed a unimodal curve but with a non-positive
- skew (Fig. 2A). 55 strains with EC₅₀ values in the range of 0.04-0.40 µg/mL showed the highest
- 141 frequency (67.07%). When further analysis the frequency distribution of these 54, a unimodal
- curve with a positive skew was constructed (Fig. 2B). The average EC₅₀ value of 0.2328 µg/mL
- for these subset of 55 F. pseudograminearum isolates was preliminarily determined as the
- baseline sensitivity for Tebuconazole of *F. pseudograminearum*, 2 low resistance (LR) isolates
- was observed from those we tested.
- The strains with LR were collected form Tangshan and Shijiazhuang respectively. Strains from
- Hengshui showed the lowest sensitivity variation on Tebuconazole, while strains from Tangshan
- sowed the highest (Table 2). There was significantly different (p>_-0.01) while with low
- 149 correlation (r=0.402) between Fludioxonil and Tebuconazole (Fig. 3), that is, there was no cross-
- resistance between the two agents.

Discussion

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- Baseline sensitivity data of a phytopathogenic fungus to a fungicide are useful to evaluate the
- risk of resistance development in sensitive populations of the fungi (Zhang et al. 2015a).
- 155 Fludioxonil belongs to the phenylpyrrole class of chemistry and has a unique mode of action, it
- inhibits the phosphorylation of glucose, thus results in the inhibition of the growth of fungal
- mycelium, which can also increase the seed emergence rate (Hysing and Wiik, 2014), it is

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158 commercialized in 2013 in China. In this study, the EC<sub>50</sub> of Fludioxonil to F.
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- 159 pseudograminearum ranged from 0.0165 to 0.1789 μg/mL, the differences may be related to the
- natural differences of the strains in different regions, the physiological differences in the strains
- themselves, as well as the population structure of the strains of F. pseudograminearum under
- 162 control level in each wheat region (Feng et al. 2020). In addition, F. pseudograminearum isolates
- 163 from different regions were clustered in the same group based on EC₅₀, indicating that the
- sensitivity of F. pseudograminearum to Fludioxonil was independent of the source and
- 165 geographical location of the strain.
- 166 Fungal pathogens may develop resistance to different fungicides under certain selection
- pressures or under conditions of adversity (Feng et al. 2020). Resistance to Fludioxonil has been
- reported in a broad range of plant pathogenic fungi such as Colletotrichum gloeosporioides from
- fruit (Schnabel et al. 2021), Sclerotinia sclerotiorum from oilseed rape (Kuang et al. 2011),
- 170 Botrytis cinerea from apple (Zhao et al. 2010) and strawberry (Fernandez-Ortuno et al. 2016), as
- well as Fusarium (Peters et al. 2008). In this research, the variation factor between the most
- sensitive strain and the least sensitive strain was 10.42, indicating that F. pseudograminearum
- was sensitive to Fludioxonil in nature. Based on the baseline sensitivity in this study (0.0610
- 174 μg/mL), there was no Fludioxonil resistant *F. pseudograminearum* isolate detected. The baseline
- sensitivity could be used for monitoring any future sensitivity shifts to Fludioxonil in the field
- populations of *F. pseudograminearum*. Meanwhile it provides evidence to suggest efficient
- fungicides and future methods for control of fungicide-resistant mutants.
- 178 Triazole fungicide, Tebuconazole and Difenoconazole for instance, is fungicide with the
- 179 characterization of high efficiency, wide spectrum, safety, long duration, strong internal
- absorption. It belongs to a sterol 14α- demethylase inhibitors (DMIs), and affects ergosterol
- biosynthesis, DMIs has been considered the most effective fungicide for the control of diseases
- caused by *Fusarium* spp. (Delen, 2016; Hellin et al., 2017). They can also prevent the formation
- of mycotoxins produced by F. culmorum and F. graminearum (Shah et al., 2018). It has been
- used for many years in the USA and Europe as well as in China. The registered commercial
- agents with Tebuconazole as active ingredient including Raxil (6% Tebuconazole), Liangshi
- 186 (1.1% Tebuconazole·19.9% Imidacloprid), Oberi (1.1% Tebuconazole·30.8% Imidacloprid).
- 187 They have been applied to control diseases including sharp eyespot, Fusarium head blight,
- powdery mildew etc. for many years, it also registered in FCR control by seed dressing. DMI
- resistant fungal pathogens have been reported in populations of *Botrytis cinerea* (Zhang et al.
- 190 2020), Pseudocercospora fijiensis (Chong et al., 2021), F. graminearum (de Chaves et al., 2022),
- 191 Monilinia fructicola (Lesniak et al. 2020), Cercospora beticola (Trkulja et al. 2017), Venturia
- 192 nashicola (Ishii et al. 2021). A low LR frequency (2.44%) on F. pseudograminearum detected in
- this research implied that rotational and substitution strategy for fungicides with other site of
- action should be implemented to delay development of serious resistance.
- Much should be done to reduce the use of fungicides in controlling of FCR, such as providing
- detailed information (active ingredient, potential targets and risk exposures) for different types of
- 197 pesticides used for seed treatments (Lamichhane and Laudinot. 2020). Clarifying cross-resistance

- of a pathogen to different fungicides will help to provide a theoretical basis for scientific application of fungicides on the control of the pathogen (Feng et al. 2020). Based on our results,
- 200 there is no cross-resistance between Fludioxonil and Tebuconazole. Population of *F*.
- 201 pseudograminearum was most sensitive to Fludioxonil in vitro. The high variation factor of
- Tebuconazole (50.21) implied that there may be different control level on wheat disease in
- 203 different wheat region. In the meantime, low resistance isolates from the field population to
- Tebuconazole implied that further consideration should be done to prohibit Tebuconazole as
- active gradient in wheat dressing agent. Our former research also indicated that Raxil and
- 206 Dividend (Difenoconazole as active ingredient) showed lower control efficacy in the field
- 207 compared with Celest (2.5% Fludioxonil) (unpublished data). Applying Fludioxonil in mixtures
- with newly fungicides, such as pydiflumetofen or biocontrol agents, other than Triazole
- 209 fungicide, maybe generally applicable for control of FCR and fungicide resistance management
- 210 of *F. pseudograminearum*.

Conclusions

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- 213 This is the first report about the baseline sensitivity of *F. pseudograminearum* populations from
- 214 China to Fludioxonil and Tebuconazole. The fungicide with active ingredients of Fludioxonil can
- be applied reasonable in the control of wheat crown rot in recent years. The baseline sensitivity
- 216 (0.0610 μg/mL for Fludioxonil, 0.2328 μg/mL for Tebuconazole) constructed in this study can
- be used to detect the resistance level of field populations in the future, so we can provide
- 218 guidance for rational use of fungicides.

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