

# Exploration of fungicide resistance to Fludioxonil and Tebuconazole of *Fusarium pseudograminearum*, the causal agent of wheat Fusarium crown rot

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## Abstract

The damage caused by wheat soil-borne diseases, especially Fusarium crown rot (FCR), affects the yield and quality of wheat increasingly. In the year 2020, a total of 297 *Fusarium pseudograminearum* strains were isolated and purified from diseased FCR wheat samples in Hebei Province. Baseline sensitivity of *F. pseudograminearum* to Fludioxonil ( $0.0610 \pm 0.0367$   $\mu\text{g/mL}$ ) and Tebuconazole ( $0.2328 \pm 0.0840$   $\mu\text{g/mL}$ ) were constructed based on in vitro tests of 61 and 82 strains, respectively. No resistance isolate to Fludioxonil was detected, while 2 low resistance isolates to Tebuconazole were detected based on the resistance index analysis. There was no cross-resistance between Fludioxonil and Tebuconazole. This study provides theoretical and practical value to monitoring the resistance of *F. pseudograminearum* to fungicides and control of FCR.

## Introduction

Fusarium crown rot (FCR), a soil-borne disease, causes major devastating diseases of wheat (*Triticum aestivum* L.) worldwide. The occurrence of FCR has been reported in many arid and semi-arid wheat-growing continents and countries including Australia (Magee, 1957), America (Smiley, 2005), Africa (Gargouri et al., 2011), Europe (Agustí-Brisach, 2018), the Middle East (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 three internodes or the production of white blighted heads and abortive seeds in the case of a severe attack (Scherm et al., 2013). As a consequence, significant yield losses are reported all over the world.

39 Hebei province, among the wheat-corn rotation region in the North China Plain, accounts for  
40 about 10% and 11.4% ~~on~~ of 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  
43 pathogenicity in China (Deng et al. 2020). This species has been repeatedly reported to be  
44 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~~ this agent ~~including~~ include  
49 Tebuconazole, Difenoconazole, and Fludioxonil. Chemical control of FCR exhibits the most  
50 effective method to limit disease, but repeated fungicidal applications reduced sensitivity to  
51 strains of *Fusarium* spp. when fungicides were employed, thus increase the risk of severe plant  
52 disease and bring a series of problems such as environmental pollution and residual toxicity.  
53 Determination of the susceptibility of Fusarium pathogen to fungicides in wheat most focused on  
54 the prevalent *F. graminearum* causing Fusarium head blight (FHB) (de Chaves et al. 2022;  
55 Breunig and Chilvers, 2021). Yin et al (2021) showed that Carbendazim had a strong inhibitory  
56 effect on *F. pseudograminearum* population from Henan, China, with a baseline sensitivity of  
57 (0.755±0.336) µg/mL. Little information is available about the activity and risk of resistance to  
58 Fludioxonil and Tebuconazole in *F. pseudograminearum*. Consequently, this study aimed to  
59 evaluate the susceptibility and cross-resistance of *F. pseudograminearum* from field populations  
60 to Fludioxonil and Tebuconazole. The results will provide a reference for the resistance  
61 monitoring of FCR predominant pathogen and the rational application of Trizole and Fludioxonil  
62 in the control of wheat crown rot in Hebei, China, and/or all over the world.

## 63 **Materials & Methods**

### 64 ***F. pseudograminearum* isolates collection**

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

### 71 **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  
75 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  
77 a.i./mL, Tebuconazole with concentration of 0.025, 0.1, 0.4, 1.6, 6.4 µg a.i./mL, PDA plates  
78 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 ~~the~~ sensitivity test, at least 7 isolates from each wheat geographic region were randomly  
81 selected to form a subset ~~population~~population. 61 *F. pseudograminearum* isolates were assessed  
82 to Fludioxonil and 82 isolates to Tebuconazole by ~~the~~ mycelial growth rate method (Secor and  
83 Rivera, 2012). In short, 0.7 cm mycelial plugs from the edge of pre-cultured colonies were  
84 transferred upside down onto the center of PDA plates amended with Fludioxonil. Mean radial  
85 mycelial growth was measured for each treatment by criss-cross after 3-4 days of incubation at  
86 27°C in the dark. Effective concentration for 50% growth inhibition (EC<sub>50</sub>) was calculated using  
87 the fungicide concentrations and the corresponding inhibition rate of mycelial growth.

88 Colony diameter (cm) = measured colony diameter-fungal plug diameter (0.7 cm)

89 Relative inhibition (%) = [(colony diameter of control - colony diameter of treatment)/colony  
90 diameter of control] × 100

91 Fungicide concentration (µg/mL) converted into a base-10 logarithmic value ( $x$ ) and inhibition  
92 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 ( $y = a + bx$ )  
94 and the correlation coefficient ( $r$ ) (Stein and Kirk, 2003).

95 The baseline sensitivity was established using the frequency distribution of EC<sub>50</sub> values (Hu et  
96 al. 2020).

## 97 **Fungicides resistant strains and their occurrence frequency**

98 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, samples classified LR, MR and HR were all taken as fungicide resistant strains.

101 Resistance index (RI) = EC<sub>50</sub> of the tested isolate/ baseline sensitivity

102 Sensitive strain (S):  $0 < RI \leq 5$ , Low resistance strain (LR):  $5 < RI \leq 10$ , Middle resistance strain  
103 (MR):  $10 < RI \leq 40$ , High resistance strain (HR):  $40 < RI$ .

104 Frequency of resistant strains (%) = (resistant strains/tested whole strains) × 100

## 105 **Cross-resistance analysis**

106 A subset of 54 *F. pseudograminearum* isolates ~~were-was~~ used to assess their ~~eross-cross-~~  
107 resistance. The linear regression analysis was carried out by using EC<sub>50</sub> of Fludioxonil to strain  
108 as X-axis and EC<sub>50</sub> of Tebuconazole to strain as Y-axis, the linear regression equation  $y = bx + a$   
109 was constructed. According to the determination coefficient (R<sup>2</sup>), the significance level of  
110 independent sample ~~T-T~~-test ( $P$  value), and Pearson correlation analysis, ~~eross-cross-~~resistance  
111 between Fludioxonil and Tebuconazole were analyzed.

## 112 **Data analysis**

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

118 LSD test ( $P \leq 0.05$ ). Pearson correlation analysis was carried out by SPSS 21., and Duncan's new  
119 complex range method was used to test the significance of differences.

## 120 Results

### 121 Sensitivity of mycelial growth of *F. pseudograminearum* to Fludioxonil

122  $EC_{50}$  values for all the 61 isolates were combined to establish a sensitivity baseline. The  $EC_{50}$   
123 values of the corresponding isolates for mycelial growth assays were continuous, range-ranging  
124 from 0.0165 to 0.1789  $\mu\text{g/mL}$ , with a mean value of  $(0.0610 \pm 0.0367)$   $\mu\text{g/mL}$  (Fig. 1A). The  
125 variation factor (the ratio of the maximum to the minimum  $EC_{50}$  values) was 10.84. Based on the  
126  $EC_{50}$  value of the tested strains, the frequency distribution showed a unimodal curve with a  
127 positive skew (Fig. 1B). The strains with  $EC_{50}$  values are in the range of 0.03-0.06  $\mu\text{g/mL}$   
128 showed the highest frequency (55.74%). The average  $EC_{50}$  value of 0.0610  $\mu\text{g/mL}$  was  
129 preliminarily determined as the baseline sensitivity for Fludioxonil of *F. pseudograminearum*.  
130 No resistance isolate of *F. pseudograminearum* was observed in the field subset population from  
131 those we tested.

132 The mean  $EC_{50}$  values of *F. pseudograminearum* strains collected from different geographic  
133 regions were with-significantly different (Table 1). The strains with the most sensitivity ( $<0.03$   
134  $\mu\text{g/mL}$ ) were formed-from Shijiazhuang, Baoding, and Cangzhou of Hebei, China. The strains  
135 with the highest  $EC_{50}$  were from Shijiazhuang and Handan. Strains from Baoding showed the  
136 lowest sensitivity variation on Fludioxonil, while strains from Shijiazhuang showed the highest.

### 137 Sensitivity of mycelial growth of *F. pseudograminearum* to Tebuconazole

138 The  $EC_{50}$  values of 82 isolates for mycelial growth assays to Tebuconazole were continuous,  
139 range-ranging from 0.0417 to 1.5072  $\mu\text{g/mL}$ . The variation factor was 50.21. Based on the  $EC_{50}$   
140 value of the tested strains, the frequency distribution showed a unimodal curve but with a non-  
141 positive skew (Fig. 2A). 55 strains with  $EC_{50}$  values in the range of 0.04-0.40  $\mu\text{g/mL}$  showed the  
142 highest frequency (67.07%). When further analysis of the frequency distribution of these 54, a  
143 unimodal curve with a positive skew was constructed (Fig. 2B). The average  $EC_{50}$  value of  
144 0.2328  $\mu\text{g/mL}$  for these subsets s of 55 *F. pseudograminearum* isolates was preliminarily  
145 determined as the baseline sensitivity for Tebuconazole of *F. pseudograminearum*, 2 low  
146 resistance (LR) isolates was observed from those we tested.

147 The strains with LR were collected from-from Tangshan and Shijiazhuang respectively. Strains  
148 from Hengshui showed the lowest sensitivity variation on Tebuconazole, while strains from  
149 Tangshan showed the highest (Table 2). There was a significantly different ( $p > 0.01$ ) while-with  
150 a low correlation ( $r=0.402$ ) between Fludioxonil and Tebuconazole (Fig. 3), that is, there was no  
151 cross-resistance between the two agents.

152

## 153 Discussion

154 Baseline sensitivity data of a phytopathogenic fungus to a fungicide are useful to evaluate the  
155 risk of resistance development in sensitive populations of the fungi (Zhang et al. 2015a).

156 Fludioxonil belongs to the phenylpyrrole class of chemistry and has a unique mode of action, it  
157 inhibits the phosphorylation of glucose, thus results-resulting in the inhibition of the growth of

158 fungal mycelium, which can also increase the seed emergence rate (Hysing and Wiik, 2014), it is  
159 commercialized in 2013 in China. In this study, the EC<sub>50</sub> of Fludioxonil to *F.*  
160 *pseudograminearum* ranged from 0.0165 to 0.1789 µg/mL, the differences may be related to the  
161 natural differences of the strains in different regions, the physiological differences in the strains  
162 themselves, as well as the population structure of the strains of *F. pseudograminearum* under  
163 control level in each wheat region (Feng et al. 2020). In addition, *F. pseudograminearum* isolates  
164 from different regions were clustered in the same group based on EC<sub>50</sub>, indicating that the  
165 sensitivity of *F. pseudograminearum* to Fludioxonil was independent of the source and  
166 geographical location of the strain.

167 Fungal pathogens may develop resistance to different fungicides under certain selection  
168 pressures or under conditions of adversity (Feng et al. 2020). Resistance to Fludioxonil has been  
169 reported in a broad range of plant pathogenic fungi such as *Colletotrichum gloeosporioides* from  
170 fruit (Schnabel et al. 2021), *Sclerotinia sclerotiorum* from oilseed rape (Kuang et al. 2011),  
171 *Botrytis cinerea* from apple (Zhao et al. 2010) and strawberry (Fernandez-Ortuno et al. 2016), as  
172 well as Fusarium (Peters et al. 2008). In this research, the variation factor between the most  
173 sensitive strain and the least sensitive strain was 10.42, indicating that *F. pseudograminearum*  
174 was sensitive to Fludioxonil in nature. Based on the baseline sensitivity in this study (0.0610  
175 µg/mL), there was no Fludioxonil resistant *F. pseudograminearum* isolate detected. The baseline  
176 sensitivity could be used for monitoring any future sensitivity shifts to Fludioxonil in the field  
177 populations of *F. pseudograminearum*. Meanwhile, it provides evidence to suggest efficient  
178 fungicides and future methods for the control of fungicide-resistant mutants.

179 Triazole fungicide, Tebuconazole, and Difenconazole, for instance, is a fungicide with the  
180 characterization of high efficiency, wide spectrum, safety, long duration, and strong internal  
181 absorption. It belongs to a sterol 14 $\alpha$ - demethylase inhibitors (DMIs), and affects ergosterol  
182 biosynthesis, DMIs ~~has~~ have been considered the most effective fungicide for the control of  
183 diseases caused by *Fusarium* spp. (Delen, 2016; Hellin et al., 2017). They can also prevent the  
184 formation of mycotoxins produced by *F. culmorum* and *F. graminearum* (Shah et al., 2018). It  
185 has been used for many years in the USA and Europe as well as in China. The registered  
186 commercial agents with Tebuconazole as active ingredient including Raxil (6% Tebuconazole),  
187 Liangshi (1.1% Tebuconazole·19.9% Imidacloprid), Oberi (1.1% Tebuconazole·30.8%  
188 Imidacloprid). They have been applied to control diseases including sharp eyespot, Fusarium  
189 head blight, powdery mildew, etc. for many years, it is also registered in FCR control by seed  
190 dressing. ~~DMI~~ DMI-resistant fungal pathogens have been reported in populations of *Botrytis*  
191 *cinerea* (Zhang et al. 2020), *Pseudocercospora fijiensis* (Chong et al., 2021), *F. graminearum*  
192 (de Chaves et al., 2022), *Monilinia fructicola* (Lesniak et al. 2020), *Cercospora beticola* (Trkulja  
193 et al. 2017), *Venturia nashicola* (Ishii et al. 2021). A low LR frequency (2.44%) on *F.*  
194 *pseudograminearum* detected in this research implied that rotational and substitution strategy  
195 strategies for fungicides with other sites of action should be implemented to delay the  
196 development of serious resistance.

197 Much should be done to reduce the use of fungicides in controlling ~~of~~FCR, such as providing  
198 detailed information (active ingredient, potential targets, and risk exposures) for different types  
199 of pesticides used for seed treatments (Lamichhane and Laudinot. 2020). Clarifying the cross-  
200 resistance of a pathogen to different fungicides will help to provide a theoretical basis for the  
201 scientific application of fungicides ~~on-in~~ the control of the pathogen (Feng et al. 2020). Based on  
202 our results, there is no cross-resistance between Fludioxonil and Tebuconazole. ~~Population-The~~  
203 population of *F. pseudograminearum* was most sensitive to Fludioxonil in vitro. The high  
204 variation factor of Tebuconazole (50.21) implied that there may be different control levels on  
205 wheat disease in the different wheat regions. In the meantime, low resistance isolates from the  
206 field population to Tebuconazole implied that further consideration should be done to prohibit  
207 Tebuconazole as an active gradient in wheat dressing agents. Our former research also indicated  
208 that Raxil and Dividend (Difenoconazole as an active ingredient) showed lower control efficacy  
209 in the field compared with Celest (2.5% Fludioxonil) (unpublished data). Applying Fludioxonil  
210 in mixtures with ~~newly~~ fungicides, such as pydiflumetofen or biocontrol agents, other than  
211 Triazole fungicide, may be generally applicable for the control of FCR and fungicide resistance  
212 management of *F. pseudograminearum*.

## 213 214 **Conclusions**

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

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