Sensitivity of *Puccinia graminis* f. sp. *tritici* Isolates From China to Triadimefon and Cross-Resistance Against Diverse Fungicides

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Abstract

Wheat stem rust caused by *Puccinia graminis* f. sp. *tritici* is an important wheat disease with sudden and devastating characteristics. The appearance and spread of new *P. graminis* f. sp. *tritici* races (Ug99, TKTTF, and TTTTF) have once again renewed the interest in the prevention and control of wheat stem rust. Fungicides can effectively control the epidemics of this disease in a short period of time. However, the fungal pathogen is prone to developing resistance. Therefore, we collected 89 isolates of *P. graminis* f. sp. *tritici* from four provinces in China and used the spore germination method to test the sensitivity of the isolates to fungicide triadimefon. Seven relatively triadimefon-sensitive isolates and six relatively triadimefon-resistant isolates were further tested for sensitivity to fungicides carbendazim, mancozeb, thiophanate-methyl, and

kresoxim-methyl. The results showed that the mean concentration for 50% of maximal effect of the isolates to triadimefon was 16.14 mg·liter⁻¹, and the mean resistance factor was 4.48. Only 29 isolates were resistant to triadimefon in which 27 isolates had low levels of resistance and 2 isolates had moderate levels of resistance. However, most of the 89 isolates had no resistance to triadimefon. There was a positive correlation between resistance to triadimefon and carbendazim, but there was no cross-resistance between triadimefon resistance with thiophanate-methyl or kresoxim-methyl resistance. This study provides valuable information for managing fungicide resistant isolates of *P. graminis* f. sp. *tritici*.

Keywords: fungicide resistance, stem rust, wheat

Wheat stem rust, caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Erikss. & E. Henn., is a serious disease that has historically caused large yield losses worldwide (Pardey et al. 2013). Since 1970, the disease has been effectively controlled through the successful deployment of stem rust resistance genes (e.g., *Sr31*) in commercial wheat cultivars across the world (Cao et al. 2019; Rouse et al. 2012). The emergence of new virulent races of *P. graminis* f. sp. *tritici* has overcome some resistance genes of existing wheat cultivars and caused outbreaks of stem rust, which has posed a threat to global food security (Nazari et al. 2009; Olivera et al. 2015; Singh et al. 2011). For example, races TTKSK (Ug99), TKTTF, and TTTTF are virulent to most widely used stem rust resistance genes *Sr31*, *SrTmp*, and *Sr9e* + *Sr13*, respectively, and they caused huge yield losses in eastern Africa and Sicily, Italy (Bhattacharya 2017; Food and Agricultural Organization of the United Nations [FAO] 2017; Olivera et al. 2015; Pretorius et al. 2000). Therefore, stem rust is again becoming a threat to wheat production worldwide.

Most Chinese wheat cultivars and breeding lines are highly susceptible to the races TTKSK (Ug99), TKTTF, and TTTTF of *P.*

graminis f. sp. *tritici* (Cao et al. 2019; Li et al. 2016; Xu et al. 2017, 2018). If those races spread to China, they would cause large-scale epidemics of wheat stem rust, and the disease would be controlled by intensively using chemicals. The chemical control of wheat rusts in China has mainly depended on triazole fungicides (Song and Wang 2004; Ye et al. 2015). The most widely used triazole fungicide is triadimefon, which is an efficient and broad-spectrum ergosterol biosynthesis inhibitor. However, the long-term use of this single fungicide may lead to the development of resistant *P. graminis* f. sp. *tritici* isolates (Chen et al. 1990).

Chemical resistance in fungi is a serious problem to be solved in agricultural production. It has been reported that wheat leaf rust fungus (*P. triticina* f. sp. *tritici*), stripe rust fungus (*P. striiformis* f. sp. *tritici*), powdery mildew fungus (*Blumeria graminis* f. sp. *tritici*), and other fungal pathogens have resistance to triadimefon (Cao et al. 2008; Yang et al. 2013; Ye et al. 2015). However, it remains unclear whether *P. graminis* f. sp. *tritici* is resistant to triadimefon. Because wheat cultivars grown in China are highly susceptible to stem rust, it is particularly important to evaluate the risk of triadimefon resistance in the *P. graminis* f. sp. *tritici* population in China. In the present study, *P. graminis* f. sp. *tritici* isolates collected from the main wheat regions of China from 2013 to 2015 were tested for sensitivity to triadimefon and four other commonly used fungicides (thiophanate-methyl, mancozeb, kresoxim-methyl, and carbendazim). The findings provide a reference for monitoring triadimefon resistance in *P. graminis* f. sp. *tritici*, thereby providing a theoretical basis for the rational or rotational use of fungicides.

Materials and Methods

Fungicides. The following fungicides were used in this study: 97% triadimefon (Kechuang Agricultural Chemical Co., Ltd.); 97% thiophanate-methyl (Runze Chemical Co., Ltd.); 97% mancozeb provided by Shenyang Institute of Chemical Industry; 50% kresoxim-methyl WDG (BASF Co., Ltd.); and 98% carbendazim provided by Shenyang Institute of Chemical Industry.

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***P. graminis* f. sp. *tritici* isolates and wheat cultivars.** A total of 89 single-pustule isolates (including 66 from wheat and 23 originally collected from *Berberis* spp. by Prof. Zhensheng Kang's team at Northwest Agriculture & Forestry University) from Heilongjiang, Liaoning, Sichuan, and Shaanxi provinces from 2013 to 2015 (Supplementary Table S1) were used in this study. Susceptible wheat cultivar Little Club (LC) used in this study was supplied by the College of Plant Protection, Shenyang Agricultural University.

Multiplication of *P. graminis* f. sp. *tritici* urediniospores. Seedlings of LC were grown in 12-cm-diameter porcelain pots to propagate *P. graminis* f. sp. *tritici* isolates. The leaves of 7-day-old seedlings were moistened with 0.05% Tween 20 using an atomizer. Then, urediniospores were gently smeared evenly onto the back sides of leaves with a flat toothpick and moistened again with 0.05% Tween 20. The inoculated seedlings were placed in a dew chamber for 16 to 20 h in the dark at 18 to 20°C before being transferred to a greenhouse bench at 20 ± 1°C with a photoperiod of 16 h. Urediniospores were collected 14 days postinoculation and stored in a refrigerator at 4°C before use.

Determining resistance of *P. graminis* f. sp. *tritici* to triadimefon. Spore germination was used to determine the response of each isolate to triadimefon (Gullino and Garibaldi 1986). Briefly, triadimefon was dissolved in sterile water to prepare a 600 mg·liter⁻¹ solution. After the medium had cooled to 45°C, the medium and the fungicide solution were mixed at different ratio to prepare plates with final fungicide concentrations of 3.25, 7.5, 15, 30, and 60 mg·liter⁻¹. The urediniospore suspension of *P. graminis* f. sp. *tritici* was evenly smeared with a cotton stick on the plates. Every concentration treatment had three replicates, and sterile water was used as the control. After 20 h (16 h in the dark followed by 4 h under fluorescent light) of incubation at 20 ± 1°C, the spores were placed under a microscope (Leica DM500), and their germination rates were recorded. For each replicate, the germination status of 100 spores was scored. If the length of the germ tube was greater than or equal to the short diameter of the spore, it was recorded as germinated; otherwise, it was recorded as not germinated.

Calculating the half-maximal effective concentration of the fungicides. The relative inhibition rate of spores = (1 – mean germination rate of treated spore/mean germination rate of control spore) × 100%.

The inhibition percentage of each treatment was calculated, and it was converted into the probability value (y) using the table of

“probability and death percentage.” Then, the regression equation was obtained through regression analysis of the logarithmic (x) and probability value (y) of each treatment concentration. When y = 5, the antilogarithm value of x was the effective inhibition of half-maximal effective concentration (EC₅₀) (Zhang et al. 2011).

Determining the baseline sensitivity of wheat to triadimefon. Because of the extensive use of triadimefon in wheat production, in nature it is not easy to collect isolates that are sensitive to triadimefon and can be used as controls. According to the literature, the EC₅₀ (3.6 mg·liter⁻¹) of the most sensitive isolate of *P. graminis* f. sp. *tritici* in 1990 was taken as the baseline sensitivity (Chen et al. 1990). According to the formula, resistance index (RI) = EC₅₀ of the tested isolate/EC₅₀ of the sensitive isolate. An RI value <5 was considered sensitive, an RI value between 5 and 10 was considered low resistant, an RI value between 10 and 40 was considered moderately resistant, and an RI value >40 was considered highly resistant. The proportion of isolates with resistance level greater than five in the whole population was treated as the resistance frequency of the pathogen.

Cross-resistance between triadimefon and different fungicides. The spore germination method (Gullino and Garibaldi 1986) was used to determine the sensitivity of seven relatively triadimefon-sensitive isolates and six relatively triadimefon-resistant isolates to four other fungicides: thiophanate-methyl, kresoxim-methyl, mancozeb, and carbendazim. The sensitivity correlations of triadimefon with carbendazim, ketoprime, thioprime, and mancozeb were determined using the EC₅₀ values by regression analysis. The significance of the correlation coefficient was used to determine whether there was cross-resistance between triadimefon and the other four fungicides.

Data analysis. Software SPSS 11.0 was used for statistical analysis, and Duncan's new multiple range test was used to test the significance of the difference.

Results

Sensitivity of *P. graminis* f. sp. *tritici* populations to triadimefon in main wheat areas. The sensitivity of 89 isolates of *P. graminis* f. sp. *tritici* from four provinces in China to triadimefon was determined. As shown in Table 1, the mean EC₅₀ value of the *P. graminis* f. sp. *tritici* isolates was 18.52 mg·liter⁻¹, and the mean resistance level was 5.14, indicating that the *P. graminis* f. sp. *tritici* population in China generally has low resistance to triadimefon.

Table 1. Half-maximal effective concentration (EC₅₀) values and mean urediniospore inhibition rates of *Puccinia graminis* f. sp. *tritici* isolates from different provinces collected in 2013 to 2015 tested with triadimefon

Location	Number	Range of EC ₅₀ (mg·liter ⁻¹)	Mean EC ₅₀ (mg·liter ⁻¹) ^z	Mean inhibition rate (%)
Yanting, Sichuan	8	17.13–28.13	23.52 a	93.47
Shaanxi	23	9.82–40.30	22.55 a	93.74
Heilongjiang	7	10.94–27.06	21.93 a	93.76
Maerkang, Sichuan	11	11.46–21.86	14.99 b	95.80
Liaoning	40	4.58–16.42	9.61 c	97.18

^z The same lowercase letter indicates no significant difference between the data ($P = 0.05$).

Table 2. Frequencies of different resistance index (RI) categories of *Puccinia graminis* f. sp. *tritici* isolates from different regions collected from 2013 to 2015 to triadimefon

Region	Frequency (%) of isolates in different RI categories			Mean RI	Highest RI ^y	Frequency (%) of resistance
	RI ≤ 5	5 < RI ≤ 10	10 < RI ≤ 40			
Shaanxi	30.43	60.87	8.70	6.26 a ^z	11.19 a	69.57
Yanting, Sichuan	0.00	100.00	0.00	6.53 a	7.81 b	100.00
Heilongjiang	14.29	85.71	0.00	6.09 a	7.52 b	85.71
Maerkang, Sichuan	90.91	9.09	0.00	4.16 b	6.07 c	9.09
Liaoning	100.00	0.00	0.00	2.66 c	4.56 d	0.00
Total	66.29	31.46	2.25	5.14	11.19	33.71

^y The highest RI = the highest half-maximal effective concentration of tested isolate/the half-maximal effective concentration of sensitive isolate.

^z The same lowercase letter means that there is no significant difference between data ($P = 0.05$).

Isolates from different areas differed in their sensitivity to triadimefon. The *P. graminis* f. sp. *tritici* isolates from Yanting, Sichuan were the most sensitive, with the mean EC_{50} value of $23.52 \text{ mg}\cdot\text{liter}^{-1}$ and a relative spore inhibition rate of 93.47%. The mean EC_{50} values of the isolates from Shaanxi; Heilongjiang; Maerkang, Sichuan; and Liaoning were 22.55, 22.45, 15.10, and $10.16 \text{ mg}\cdot\text{liter}^{-1}$, respectively, and the inhibition rates were 93.76, 93.74, 95.80, and 99.18%, respectively. Compared with the other regions, the isolates from Liaoning had the lowest mean EC_{50} value and the lowest mean inhibition rate or the highest sensitivity level ($P < 0.05$), and no fungicide-resistant isolate was found. The sensitivity of isolates in different areas of the same province was also significantly different. The mean EC_{50} value of isolates collected from Yanting, Sichuan was higher than that in the Maerkang region, which is also in Sichuan province. The sensitivity ranges of different isolates collected in the same area were also significantly different ($P < 0.05$). For example, the lowest EC_{50} value in Yanting was $17.13 \text{ mg}\cdot\text{liter}^{-1}$, whereas the highest value was $28.13 \text{ mg}\cdot\text{liter}^{-1}$ (Supplementary Table S1).

Resistance of *P. graminis* f. sp. *tritici* populations to triadimefon in the main wheat regions of China. The RI values to triadimefon in *P. graminis* f. sp. *tritici* from different regions are shown in Table 2. The results showed that about one-third of the tested *P.*

graminis f. sp. *tritici* isolates had developed some level of resistance to triadimefon. The isolates with the highest RI values were collected from Shaanxi. It is worth mentioning that some of the *P. graminis* f. sp. *tritici* isolates originally collected from *Berberis* spp. had an RI of 11.19, and the sexually reproduced isolates in general had significantly higher RI values than those collected from wheat in the other regions. The isolates collected from Liaoning were the most sensitive to triadimefon (the mean RI is 2.83), and all of the isolates were in the sensitive range ($RI < 5$). In addition, $> 90\%$ of the isolates from Maerkang were sensitive to triadimefon. The isolates from the Yanting area of Sichuan had low levels of triadimefon resistance.

Only 2 of the 89 isolates of *P. graminis* f. sp. *tritici* had a moderate level of triadimefon resistance, and they were from Shaanxi. According to the frequency distribution of resistance levels, 33.71% of the tested isolates had low to moderate levels of resistance to triadimefon, and the isolates with moderate resistance were only 2.25%. The results indicated that most isolates were not resistant to triadimefon.

Cross-resistance between triadimefon and other fungicides. The mean urediniospore inhibition rates of tested fungicides to *P. graminis* f. sp. *tritici* isolates were presented in Table 3. Triadimefon and thiophanate-methyl had high inhibition rates to tested *P. graminis*

Table 3. Half-maximal effective concentration (EC_{50}) values and mean urediniospore inhibition rates of *Puccinia graminis* f. sp. *tritici* isolates to tested fungicides

Fungicide	Range of EC_{50} ($\text{mg}\cdot\text{liter}^{-1}$)	Mean EC_{50} ($\text{mg}\cdot\text{liter}^{-1}$) ^a	Mean inhibition rate (%)
Carbendazim	29.37–85.74	56.26 a	83.49
Triadimefon	7.13–37.17	18.96 b	94.21
Thiophanate-methyl	0.51–11.57	3.77 c	90.85
Mancozeb	0.06–0.35	0.16 c	87.69
Kresoxim-methyl	0.01–0.22	0.05 c	85.26

^a The same lowercase letter indicates no significant difference between the data ($P = 0.05$).

Fig. 1. Cross-resistance between different fungicides. **A**, Cross-resistance between triadimefon and carbendazim. **B**, Cross-resistance between triadimefon and mancozeb. **C**, Cross-resistance between triadimefon and kresoxim-methyl. **D**, Cross-resistance between triadimefon and thiophanate-methyl. EC_{50} , half-maximal effective concentration.

f. sp. *tritici* isolates. The correlation coefficients of 13 isolates were all >0.94, indicating that all EC₅₀ values were reliable (Supplementary Table S2). Therefore, the results of the cross-resistance between triadimefon and the other four fungicides based on EC₅₀ were also reliable. The correlation analysis showed that there was a significant positive correlation between the sensitivities of *P. graminis* f. sp. *tritici* to triadimefon and carbendazim ($r = 0.9411$, $P < 0.001$) (Fig. 1A), indicating that most isolates were sensitive to both fungicides and that some isolates had cross-resistance to both triadimefon and carbendazim. There was also a certain correlation between the sensitivities to triadimefon and mancozeb ($r = 0.7764$, $P = 0.002 < 0.05$) (Fig. 1B). However, there was no significant correlation between the sensitivities to triadimefon and kresoxim-methyl ($r = 0.1992$, $P > 0.05$) or between triadimefon and thiophanate-methyl ($r = 0.1603$, $P > 0.05$) (Fig. 1C and D). These results indicated that there was no cross-resistance between triadimefon and thiophanate-methyl or kresoxim-methyl.

Discussion

Triazole fungicides exert high selection pressure on fungal pathogens. Because this group of fungicides has been used for a long period of time, many pathogens have developed resistance. It has been reported that fungal pathogen populations have cross-resistance to triazole fungicides and sterol demethylation inhibitors (Collins and Brown 1999). If *P. graminis* f. sp. *tritici* resistance to these fungicides becomes widespread in the field, it will bring huge losses to fungicide manufacturers and agricultural production. At present, triadimefon is still the main fungicide used for the control of wheat rusts, and a large amount of triadimefon is applied in fields every year in China. Therefore, we should pay attention to the emergence of fungicide-resistant isolates. Prolonging the effective life of triazole fungicides by delaying the development of pathogen resistance is an urgent task.

In this study, the maximum EC₅₀ value of 89 *P. graminis* f. sp. *tritici* isolates from different wheat-producing areas in China was 8.81 times the minimum EC₅₀ value. This range in EC₅₀ values might be because of the natural differences among sensitive isolates or differences in the environments that the isolates were collected from. Based on the distribution of resistance-level frequency, the triadimefon-sensitive isolates were dominant in the field, and few isolates had a moderate level of resistance, indicating that the *P. graminis* f. sp. *tritici* population in China is not resistant to triadimefon at present. This might be the reason why triadimefon has been so successful in controlling wheat stem rust over the last few decades.

In the future, it will be necessary to monitor whether *P. graminis* f. sp. *tritici* in the field is resistant to triadimefon. To achieve this, researchers should take a large number of samples from wheat fields that are often treated with triadimefon and conduct in-depth investigations. At the same time, we should follow the example of the efforts to control wheat powdery mildew by establishing a mature fungicide resistance monitoring system, carrying out field resistance monitoring every year, and determining whether fungicide-resistant isolates exist. These measures would provide a theoretical basis for managing fungicide resistance in *P. graminis* f. sp. *tritici* and provide guidance for the production and use of fungicides.

In this study, we also tested *P. graminis* f. sp. *tritici* for sensitivity to other chemicals. Triadimefon and thiophanate-methyl had high inhibition rates to the tested *P. graminis* f. sp. *tritici* isolates. Although the fungicide concentrations used in the study were lower than those used in the field, the inhibition rate of urediniospores was still very high. This is expected because urediniospores were immediately and directly put in contact with the fungicide. Under field conditions, much of the applied fungicide may drop to the ground. A higher than inhibiting rate is needed to keep an adequate concentration on plants for a much longer period of time than the period that we tested in this study. Our data indicated that there was possible cross-resistance between triadimefon and carbendazim. However, only two isolates showed some level of resistance to these fungicides. Further tests with more isolates are needed to verify cross-resistance. Practically, the mixed use or alternate use of triadimefon and carbendazim in crop

production should be avoided to preserve the control efficiency of the two fungicides. At present, there is no cross-resistance between triadimefon and thiophanate-methyl or kresoxim-methyl, and therefore, thiophanate-methyl and kresoxim-methyl may be used instead of triadimefon to control wheat stem rust in some areas where the *P. graminis* f. sp. *tritici* population is largely resistant to triadimefon. Triadimefon may also alternately used with thiophanate-methyl or kresoxim-methyl in wheat production in other areas to prolong the service life of the triadimefon and ensure that the pathogen is effectively controlled.

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