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Baseline sensitivity of *Fusarium graminearum* to difenoconazole and sensitivity correlation to other fungicides

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Abstract: *Fusarium* head blight (FHB), caused by *Fusarium graminearum*, is one of the most important destructive diseases of wheat, which leads to the decline in wheat yield and quality. Difenoconazole, a triazole fungicide, is an exemplary demethylation inhibitor of ergosterol biosynthesis with broad-spectrum and high-efficiency fungicidal activity. In this study, 107 isolates of *Fusarium graminearum* collected from Henan Province from 2016 to 2017 were assayed to determine their sensitivity to difenoconazole based on the mycelial growth inhibition by the fungicide. The results showed that difenoconazole had a high inhibitory effect on the mycelial growth of *F. graminearum*, and the EC_{50} value was in range of 0.012 8 to 0.607 9 mg/L with a normal distribution, and the mean EC_{50} value was (0.223 9 ± 0.119 2) mg/L (mean ± SD). Spearman's rho (ρ) for the $\log_{10}$ of the EC_{50} values of 20 isolates of *F. graminearum* between difenoconazole and epoxiconazole, carbendazim, phenamacril, pydiflumetofen, tebuconazole, prothioconazole, and metconazole were analysed. The sensitivity correlation analysis showed that there was low level correlation between difenoconazole and metconazole, but no correlation with other tested fungicides.Therefore, these sensitivity data could be used as the baseline sensetivity of *F. graminearum* to difenoconazole in Henan Province and provide reference for the sensitivity monitoring to difenoconazole in the *F. graminearum* population and fungicide reasonable application.

Keywords: *Fusarium graminearum*; difenoconazole; baseline sensitivity; correlation analysis of fungicides

禾谷镰孢菌对苯醚甲环唑的敏感性基线及与其他杀菌剂敏感性的相关性分析

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摘　要：由禾谷镰孢菌引起的赤霉病是小麦上的重要病害，可严重影响小麦的产量并降低小麦的品质。苯醚甲环唑属于三唑类杀菌剂，是甾醇脱甲基化抑制剂，具有较高的抑菌活性。采用菌丝生长速率法测定了于2016—2017采集自河南省的107株禾谷镰孢菌对苯醚甲环唑的敏感性。结果表明：苯醚甲环唑对禾谷镰孢菌菌丝的生长具有较强的抑制效果，其有效抑制中浓度(EC_{50})值范围为0.0128~0.6079 mg/L，符合正态分布，平均EC_{50}值为(0.2239 ± 0.1192) mg/L。因此，这些敏感性数据可以作为河南省禾谷镰孢菌对苯醚甲环唑的敏感性基线。通过对苯醚甲环唑与其他7种杀菌剂氟环唑、多菌灵、氰烯菌酯、氟唑菌酰羟胺、戊唑醇、丙硫菌唑及叶菌唑对20株禾谷镰孢菌的$\log_{10} EC_{50}$值之间的Spearman's rho (ρ)相关性分析发现：苯醚甲环唑与叶菌唑之间具有较低水平的相关性，与其他供试杀菌剂之间无相关性。本研究可为监测河南省禾谷镰孢菌对苯醚甲环唑的抗药性发展和防控小麦赤霉病合理用药提供依据。

关键词: 禾谷镰孢菌；苯醚甲环唑；敏感性基线；杀菌剂相关性分析

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0 Introduction

In recent years, *Fusarium* head blight (FHB) has widely occurred in countries and regions such as Asia, Africa, Europe, Australia, and the United States[1-2], and has long been a noteworthy question in the agricultural field. As a cosmopolitan disease, it reduces the quality and yield of cereals including wheat, maize, oat and so on[3-5]. Studies have reported that *Fusarium graminearum* can also be isolated from weeds, soybeans and other non-grain hosts[6-7]. When the disease occurs, the ascospores and conidia produced by *F. graminearum* are spread by airflow and rainwater to infect crops[8]. In the case of wheat, FHB is capable of infecting all parts of it, with ear rot being the most serious, resulting in 10%-20% reduction in wheat yield, even 40%, or even no harvest in pandemic years. In addition to losing the seed and industrial value, contaminated wheat kernels can also produce a variety of trichothecene derivatives including deoxynivalenol (DON), nivalenol (NIV) and certain secondary metabolites[9-12]. Once consumed by humans and animals, it easily causes poisoning, which seriously threatens health, safety and even life[13-15]. Along with the change of global climate and the influence of tillage system such as increasing the area of straw returning to the field, the microclimate of field planting has also changed, resulting in a substantial increase in the frequency and scope of the disease. FHB is primarily relevant to the *F. graminearum* species complex (FGSC), such as *F. asiaticum*, *F. graminearum*, *F. verticillioides*, *F. equiseti*, *F. meridionale*, *F. vorosii*, *F. boothii*, and *F. brasilicum*[10-12, 16-18].

In China, the occurrence of FHB was first reported in 1936. It has spread from the north to the west since 2010. it has spread from the north to the west[19]. The incidence of diseases in the northwestern wheat region has also increased significantly. The areas with frequent occurrence of wheat head blight are mainly concentrated in the winter wheat areas of the Huanghuai River Basin, the Yangtze River Basin and South China[11, 20]. Such diseases also occur in the spring wheat areas of Northeast China. Worse still, the toxin content of wheat scab in some areas even exceeds the present national limit. *F. graminearum* and *F. asiaticum* are the most available pathogens in China and the former is the dominant species in Henan Province[21-22]. Henan Province is located in east-central China, the middle and lower reaches of the Yellow River, with a humid to semi-humid, warm temperate to subtropical monsoon climate. Known as the "granary of China", Henan's annual wheat output accounts for more than a quarter of the country's total, making it the largest wheat producing province in China[20]. Therefore, it is particularly crucial to prevent and control wheat diseases.

Epidemiological studies have shown that it is extremely important to control wheat diseases during the initial infection, and this requires the control of *F. graminearum* before its occurrence. For the time being, due to the lack of wheat disease-resistant varieties, chemical control is still the most effective method in the treatment of FHB. Spraying fungicides during the flowering period of wheat is used for field control[23-24]. Examples include carbendazim (benzimidazoles), tebuconazole (demethylation inhibitors, DMIs) and pydiflumetofen (succinate dehydrogenase inhibitors, SDHIs). However, some fungicides such as carbendazim and tebuconazole have been used to control FHB for many years. With the increasing number and amount of application, a growing body of research has shown that long-term and single use of fungicides possibly give rise to a series of serious problems, such as increased *F. graminearum* resistance resulting in an obvious decrease in its effectiveness against target diseases, chemical residues that are difficult to degrade, and environmental pollution which is a threat to biological health[25]. Therefore, we demand to seek new fungicides for controlling efficiently wheat head blight. In the meantime, the neo-fungicides were needed to compare with the fungicides in common use to determine the sensitivity relationship between them.

Difenoconazole, an excellent triazole fungicide developed by Syngenta, is capable of destroying the structure and function of cell membranes by inhibiting the biosynthesis of cellular ergosterol, thereby preventing the growth of pathogenic fungi. It is a high-efficiency and low-residue sterol DMI fungicide[26]. Moreover, since difenoconazole was launched in 1989, it has shown strong systemic properties and a broad range of antifungal spectrum like other DMI fungicides[27]. It is effective against a variety of fungal diseases including Ascomycotina, Basidiomycotina, Deuteromycotina in apple, wheat, tobacco and other plants, which exhibit high protective and therapeutic effects[28-31]. With regard to the prevention and control of wheat diseases, as of 2020, the most commonly registered control targets of single-agent difenoconazole were *Gaeumannomyces graminis* and *Ustilago tritici* (http://www.chinapesticide.org.cn). Nevertheless, the single-agent registration of *F. graminearum* has not yet been discovered.

As a consequence, to evaluate the sensitivity of *F. graminearum* field isolates to difenoconazole and clarify the sensitivity correlation between difenoconazole and other fungicides, this study aimed to: (a) determine the EC_{50} values of *F. graminearum* field isolates collected in different wheat regions of Henan Province and establish the baseline sensitivity to difenoconazole. (b) evaluate the sensitivity correlation relationship between difenconazole and each of the fungicides (epoxiconazole, carbendazim, phenamacril, pydiflumetofen, tebuconazole, prothioconazole, and metconazole).

1 Materials and methods

1.1 Isolate collections

When the disease characteristics of wheat ears were remarkable in late April, 107 field isolates were collected from 11 prefecture-level cities in Henan Province from 2016 to 2017 (Kaifeng, Luohe, Nanyang, Pingdingshan, Shangqiu, Xinxiang, Xinyang, Xuchang, Zhengzhou, Zhoukou, and Zhumadian). A few infected ears were sampled randomly from each wheat field and each field was more than 10 kilometers apart. Under a sterile environment, individual wheat grains with obvious FHB symptoms were washed twice in sterile water and then transferred to 0.1% (*V*/*V*) sodium hypochlorite solution for 30 s sterilization in the first place. Subsequently, sterilized kernels were rinsed 2-3 times with sterile water. In the end, after being dried with sterile filter paper, all samples were inoculated on potato sucrose agar (PSA, 200 g fresh potato, 20 g sucrose and 15 g agar, boiled and filtered, then diluted to 1 L distilled water) plate and grown under dark conditions for 2-3 days at 25 °C. In order to obtain pure strains, the single-spore isolation technique was performed on the cultures. Using a 5 mm diameter puncher, ten mycelial disks on the edge of the colony grown for 3 days, were added to 100 mL mung bean soup medium (30 g mung beans were boiled in 1 L distilled water for 20 min and residue was removed) and cultured at 25 °C, 120 r/min, dark and light alternately shaken for 7 days. Thereafter, we used double-layer sterile lens cleaning paper to filter, and evenly spread the appropriately diluted filtrate on a water agar (20 g agar per litre of distilled water) glass slide, (it was advisable to observe a single spore at high power), and transferred the single spore to the PSA plate at 25 °C in the dark for 3 days. The isolates of *F. graminearum* were cultured in PSA slants and stored at 4 °C until subsequent experiments.

1.2 Fungicides

All the dosage forms of fungicides in this study were technical-grade original fungicides. All tested fungicides were dissolved to obtain 10 mg/mL stock solution and stored at 4 °C. The information about these fungicides are shown in Table 1.

Table 1 Information of tested fungicides in the study

Fungicide	Concentration	Solvent	Source
difenoconazole	97%	Methanol	Sinon Chemical (China) Co., Ltd. (Shanghai, China)
epoxiconazole	97%	Acetone	the Jiangsu Fengdeng crop protection Co., Ltd. (Changzhou, China)
carbendazim	98%	0.1 mol/L Hydrochloric acid	Jiangsu Rotam Chemistry Co., Ltd. (Suzhou, China)
phenamacril	95%	Methanol	Jiangsu Institute of Pesticide Co. Ltd. (Nanjing, China)
pydiflumetofen	98%	Methanol	Syngenta Crop Protection Co., Ltd. (Shanghai, China)
tebuconazole	97%	Methanol	Jiangsu Institute of Pesticide Co. Ltd. (Nanjing, China)
prothioconazole	95%	Acetone	Shandong Hailir Pesticides & Chemicals Co. Ltd. (Weifang, China)
metconazole	95%	Acetone	Jiangsu Huifeng Biological Agriculture Co., Ltd. (Yancheng, China)

1.3 Sensitivity to difenoconazole

In this study, the sensitivity of 107 *F. graminearum* isolates to difenoconazole was determined by the mycelial growth rate method[32]. The 50% effective concentration (EC_{50}) required to inhibit mycelial growth was calculated, and it was conducive to analyze the frequency distribution range of EC_{50} value. In this study, 5 mm mycelial disks from the edge of the colonies pre-cultured for 3 days were inoculated on PSA plates containing gradient concentration difenoconazole of 0, 0.1, 0.2, 0.4, 0.6 and 0.8 mg/L and the corresponding concentration of methanol for control treatment (the solvent effect was insignificant and negligible, data not shown). After being cultured at 25 °C for 3 days, the mean diameter of the fungal colonies was measured by criss-cross. The inhibition rate of difenoconazole on mycelial growth was calculated by the formula as previously described[17]. Next, the logarithm of the concentration was taken as the value on the X axis, and the probability value of the inhibition rate was taken as the value on the Y axis, and then the linear regression equation and EC_{50} value of *F. graminearum* to difenoconazole were calculated. Three replicates were set for each treatment and the experiment was repeated three times.

1.4 Sensitivity correlation analysis

A total of 20 isolates were selected from all the *F. graminearum* isolates used above for sensitivity tests as previously described, to demonstrate the sensitivity relationship between difenoconazole and the other seven fungicides (epoxiconazole, carbendazim, phenamacril, pydiflumetofen, tebuconazole, prothioconazole, and metconazole). Four cities were selected from northern Henan to southern Henan and 4-7 isolates were randomly selected from each city among all the isolates from 2016 to 2017. The isolates used in the experiment were numbered as follows: KF1721, KF1723, KF1725, KF1729, XC1710, XC1716, XC1718, KF1618, KF1628, KF1630, NY1604, NY1613, NY1614, NY1615, NY1620, XC1629, ZMD1620, ZMD1623, ZMD1625 and ZMD1634. The sensitivities of the *F. graminearum* isolates to the test fungicides were determined based on mycelial growth inhibition, as described above (Table 2). The solvent controls were treated likewise (the solvent effect was insignificant and negligible, data not shown). The sensitivity correlation between difenoconazole and the other seven fungicides were analyzed by Spearman's rank correlation. Spearman's rho (ρ) for the $\log_{10}$ of the EC_{50} values of 20 isolates of *F. graminearum* between difenoconazole and tested fungicides were analysed[18, 33]. Three replicates were set for each treatment and the experiment was repeated three times.

Table 2 Concentrations of tested fungicides in the study

Fungicide	Concentrations/(mg/L)
difenoconazole	0, 0.1, 0.2, 0.4, 0.6 and 0.8
epoxiconazole	0, 0.05, 0.1, 0.2, 0.4 and 0.8
carbendazim	0, 0.1, 0.3, 0.5, 0.7 and 0.9
phenamacril	0, 0.05, 0.1, 0.2, 0.4 and 0.8
pydiflumetofen	0, 0.006 25, 0.025, 0.1, 0.4 and 0.8
tebuconazole	0, 0.025, 0.1, 0.4, 0.8 and 1.6
prothioconazole	0, 0.312 5, 0.625, 1.25, 2.5 and 5.0
metconazole	0, 0.012 5, 0.025, 0.05, 0.1, 0.2 and 0.4

1.5 Statistical analysis

All data were analyzed using IBM SPSS Statistics v20 (SPSS Inc., Chicago). The mean value of EC_{50} was tested by Fisher's least significant difference (LSD, $P = 0.01$). The $\log_{10} EC_{50}$ value of difenoconazole is on the X-axis and the $\log_{10} EC_{50}$ values of the other seven fungicides are on the Y-axis, respectively.

2 Results and analysis

2.1 Sensitivity to difenoconazole

The sensitivity of 107 *F. graminearum* isolates in Henan Province to difenoconazole was determined based on mycelial growth. The results indicated that the EC_{50} value range of difenoconazole in 2016-2017 was 0.012 8-0.607 9 mg/L, and the mean $EC_{50} \pm SD$ value was (0.223 9 ± 0.119 2) mg/L (Table 3).

Table 3 Sensitivity of *Fusarium graminearum* to difenoconazole in Henan Province from 2016 to 2017

Source	Number of isolates			EC_{50} value range/(mg/L)[1)]	Average EC_{50} value/(mg/L)[2)]
	2016	2017	Total		
Kaifeng	5	16	21	0.073 6-0.607 9	0.229 0 ± 0.133 5 ab
Luohe	0	2	2	0.087 6-0.311 2	0.199 4 ± 0.158 1 ab
Nanyang	10	0	10	0.105 7-0.357 3	0.254 4 ± 0.075 2 ab
Pingdingshan	6	0	6	0.154 0-0.339 6	0.243 1 ± 0.073 1 ab
Shangqiu	0	8	8	0.088 0-0.358 0	0.192 5 ± 0.092 6 ab
Xinyang	6	0	6	0.153 0-0.566 2	0.393 3 ± 0.168 1 a
Xinxiang	4	1	5	0.117 2-0.266 6	0.222 3 ± 0.060 2 ab
Xuchang	6	7	13	0.012 8-0.331 7	0.132 7 ± 0.102 7 b
Zhengzhou	9	8	17	0.030 2-0.467 6	0.206 0 ± 0.120 4 ab
Zhoukou	6	3	9	0.059 3-0.387 7	0.201 6 ± 0.100 6 ab
Zhumadian	8	2	10	0.108 9-0.361 0	0.269 3 ± 0.082 3 ab
Total	60	47	107	0.012 8-0.607 9	0.223 9 ± 0.119 2 ab

Note: 1) Values are the mean $EC_{50} \pm SD$ of three replicate plots. 2) Means followed by the same letters are difference with no significance based on Fisher's least significant difference (LSD) test ($P = 0.01$).

By analyzing the frequency distribution range of the EC_{50} value, we found that the sensitivity curve of difenoconazole appeared in a unimodal form, and the distribution range was relatively narrow and mainly concentrated in the range of 0.1-0.3 mg/L (Fig.1).

After analyzing the geographical distribution region of the EC_{50} value, it was obvious that the EC_{50} value in the southern part of Henan Province was slightly overtopped compared to other areas (Fig.2), particularly the mean EC_{50} value (0.393 3 ± 0.168 1) mg/L of Xinyang, but there was no significant difference compared with the overall. From 2016 to 2017, the strains of *F. graminearum* in Henan Province were sensitive to difenoconazole, and no

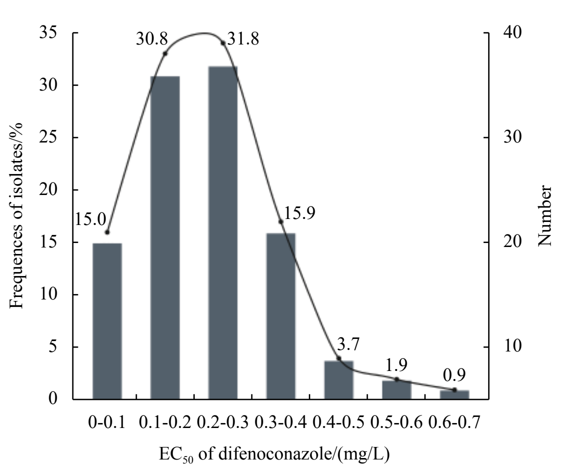
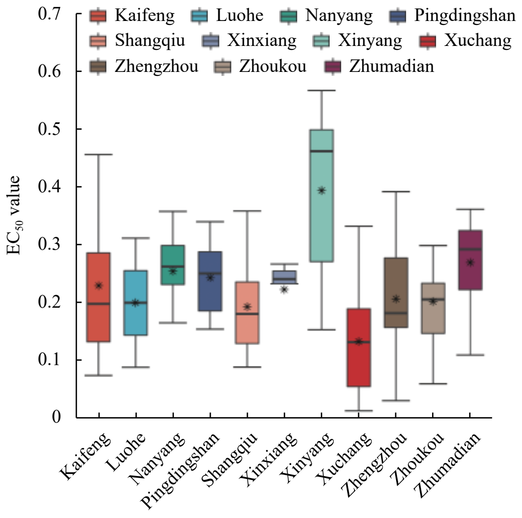


Fig. 1 The frequency distribution of EC_{50} values of 107 *F. graminearum* isolates to difenoconazole based on mycelial growth in Henan Province



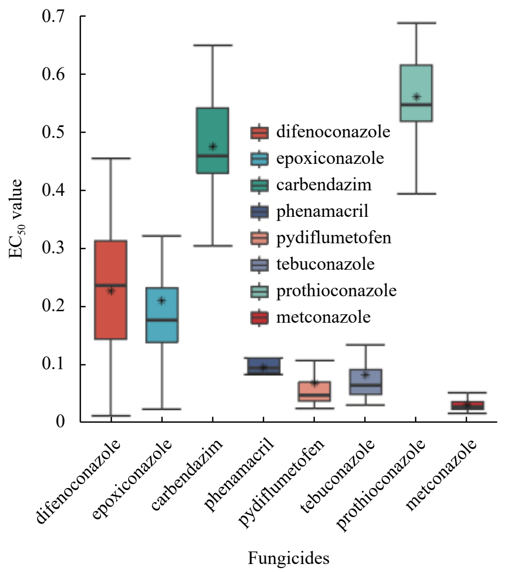
*Signified the mean EC_{50} value of each city.

Fig. 2 Sensitivity of *F. graminearum* to difenoconazole of each city in Henan Provice from 2016 to 2017

resistant isolates were found.

2.2 Sensitivity correlation analysis

To determine whether there was sensitivity correlation between difenoconazole, epoxiconazole, tebuconazole, prothioconazole, metconazole, carbendazim, phenamacril and pydiflumetofen, 20 randomly selected isolates were tested for sensitivity among all *F. graminearum* isolates from 2016 to 2017. The results suggested that the mean EC_{50} values of difenoconazole, epoxiconazole, tebuconazole, prothioconazole, metconazole, carbendazim, phenamacril and pydiflumetofen were 0.227 6, 0.210 9, 0.083 9, 0.800 9, 0.032 0, 0.476 0, 0.096 0



*Signified the mean EC_{50} value of each fungicide.

Fig. 3 Sensitivity of *F. graminearum* to 8 kinds of fungicides

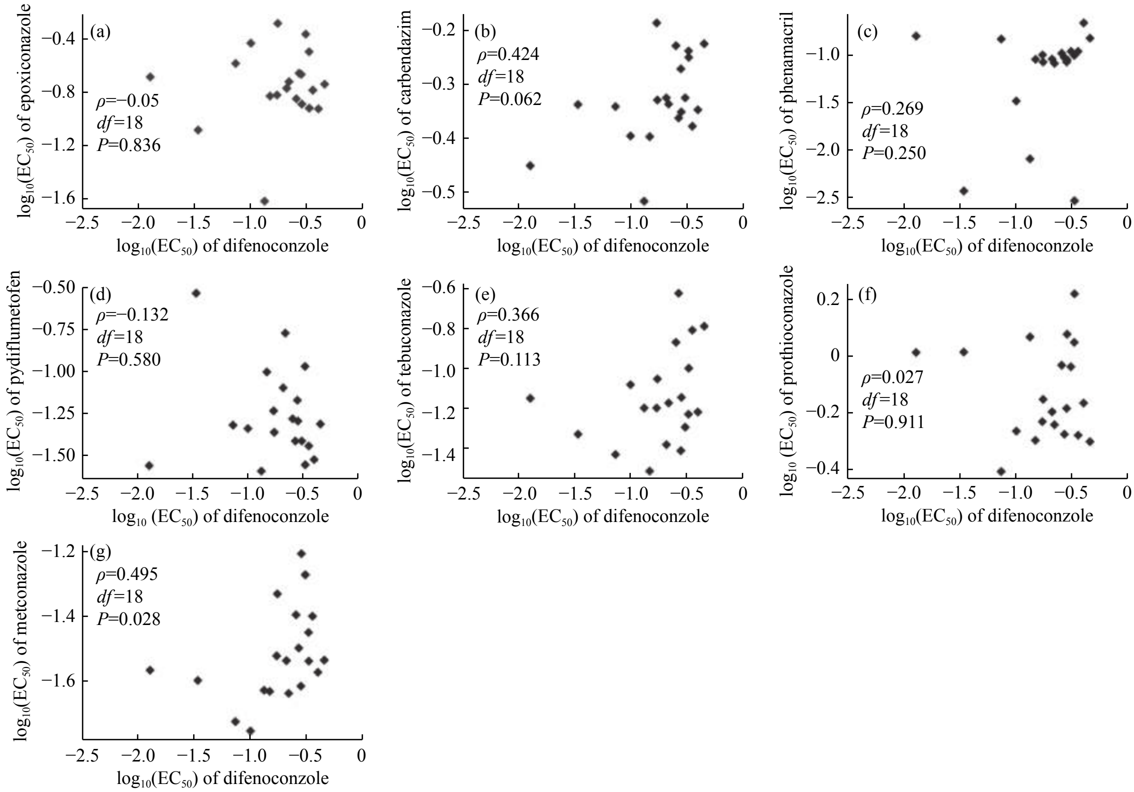
and 0.069 6 mg/L, respectively (Fig.3).

The sensitivity correlation between difenoconazole and the other seven fungicides were analyzed by Spearman's rank correlation. The Spearman's ρ for the $\log_{10}$ of the EC_{50} values of 20 isolates of *F. graminearum* between difenoconazole and epoxiconazole, carbendazim, phenamacril, pydiflumetofen, tebuconazole, prothioconazole and metconazole were −0.05 ($P > 0.05$), 0.424 ($P > 0.05$), 0.269 ($P > 0.05$), −0.132 ($P > 0.05$), 0.366 ($P > 0.05$), 0.027 ($P > 0.05$) and 0.495 ($P \leqslant 0.05$), respectively (Fig.4). The results showed that was low level correlation between difenoconazole and metconazole, but no correlation with other tested fungicides.

3 Conclusion and discussion

As one of the food crops humans mostly consume, wheat disease is regarded as a cosmopolitan food security problem[34]. FHB not only affects the yield and quality of wheat, but reduces the germination rate and flour output of wheat kernels[35]. Hitherto, wheat varieties with high resistance and suitable for large-scale planting have not been obtained [36-37]. Thus, chemical control is mainly adapted in production, combined with the reasonable disposal of crop stubble, suitable rotation cultivation and other control measures to reduce the occurrence of FHB to a certain extent[38]. At the moment, the commonly used fungicides are benzimidazoles, SDHIs, DMIs, cyanoacrylate esters, etc[39-40]. Unfortunately, carbendazim-resistant and tebuconazole-resistant strains have been isolated from the field in China[41]. Studies have revealed that difenoconazole is used to control pepper anthracnose, pear speck spot, tomato gray mold and other diseases[42-44], but there are few reports on the establishment of wheat head blight sensitivity baseline in China. No resistant strains were found. Therefore, determining the sensitivity of *F. graminearum* to difenoconazole plays an essential role in the identification and monitoring of resistance in Henan Province and even China.

In the present study, we detected the sensitivity of 107 wheat scab field isolates collected from different areas of Henan Province in response to



Note: The dots represent *Fusarium graminearum* isolates used for sensitivity correlation analysis; ρ, Spearman's rho; *df*, degrees of freedom and *P*, *P*-value

Fig. 4 Sensitivity correlation between difenconazole and epoxiconazole (a), carbendazim (b), phenamacril (c), pydiflumetofen (d), tebuconazole (e), prothioconazole (f) and metconazole (g) was analysed by Spearman's rank correlation coefficient for $\log_{10}$-transformed EC_{50} values

difenoconazole. The range of EC_{50} values and mean were 0.0128-0.6079 mg/L and (0.2239 ± 0.1192) mg/L, respectively. It was significantly lower than the values in Rekanović's research[45]. We suspected that the utilization of such fungicide was probably continual in Serbia[45]. Our data demonstrated that, even though *F. graminearum* in Henan Province showed sensitivity to difenoconazole in overall tendency, the EC_{50} value in Xinyang was the highest, indicating that *F. graminearum* in this region was less sensitive to difenoconazole. The likely explanations might be that Xinyang is located in the southernmost point of Henan Province, south of the Huaihe River, with abundant precipitation and suitable for the propagation of FHB. On the whole, FHB in Henan Province was sensitive to difenoconazole and no resistant isolates were found. The sensitivity basically conformed to the normal distribution and the EC_{50} value was about 0.1-0.3 mg/L. As previously reported, difenoconazole plays a significant control effect on *F. graminearum*[46].

For the sake of interpreting whether there was a sensitivity correlation relationship between difenoconazole and other fungicides, we selected epoxiconazole, tebuconazole, prothioconazole, metconazole, which belonged to the DMIs, and carbendazim, phenamacril, pydiflumetofen not in the same category as the fungicides with excellent efficacy[17, 40, 47]. In previous studies, the results showed that there were significant correlations between different DMI fungicides to rice false smut[48]. However, in our study, we found that there was low level correlation between difenoconlazole and metconazole, but not with other DMI fungicides. It might be on the score of the fact that the sensitivity correlation is related to different kinds of pathogens. It may also be that DMI fungicides target different CYP51 proteins, including CYP51A, CYP51B, and CYP51C in *F. graminearum*[49]. Moreover, Beck's research had confirmed there was obvious divergence of the topology with triazolinthion-head of prothioconazole to heme, resulting in the binding mode of prothioconazole and CYP51 distinguished from the classic triazole fungicides[50]. Whether the diverse target binding sites lead to otherness in sensitivity correlation because of different structures for difenoconazole, further verifications should be considered. In the present study, we found that there was no correlation relationship between difenoconazole and other tested fungicides. We speculated that this finding was induced by the diversity in mechanism of action of the different fungicides. The mechanism of action of DMI fungicides is effected by inhibiting the biosynthesis of cellular ergosterol. The mechanism of action of benzimidazole fungicides (carbendazim) is achieved by inhibiting microtubule assembly by binding to β-tubulin. The mechanism of action of succinate dehydrogenase inhibitor fungicides (SDHIs, pydiflumetofen) is effected by interfering with the respiratory chain complex Ⅱ. Although the mechanism of cyanoacrylate fungicides (phenamacril) is undefined, it is likely related to type I myosin5[51].

Given all that, in order to deal with the risk of FHB, the application of fungicides with high-efficiency and low-toxicity is the most effective means of control at present. As far as we know, difenoconazole has a significant inhibitory effect on *F. graminearum*, and no similar reports have been found in China till now. Thus, we recommend that the baseline sensitivity of difenoconazole in this study should be regarded as a basis and the fungicide used in field production should be publicized. Moreover, it is practicable to reduce the frequency of usage to delay the occurrence of fungicide resistance like applying difenoconazole in combination with other fungicides or in rotation.

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