Quantification of *Fusarium graminearum* in Harvested Grain by Real-Time Polymerase Chain Reaction to Assess Efficacies of Fungicides on Fusarium Head Blight, Deoxynivalenol Contamination, and Yield of Winter Wheat

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ABSTRACT

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We used a real time polymerase chain reaction-based assay and visual disease assessment to evaluate the efficacies of Js399-19, tebuconazole, a mixture of tebuconazole and thiram, azoxystrobin, carbendazim, and thiram on the development of Fusarium head blight (FHB) and deoxynivalenol (DON) contamination and on the yield of winter wheat (cv. Nannong no. 9918) after artificial inoculation under field conditions with *Fusarium graminearum*. The incidence of infected spikelets (IIS), amount of *F. graminearum* DNA (*Tri5* DNA), total DON (containing DON, 3-acetyl-deoxynivalenol, and 15-acetyl-deoxynivalenol) concentration, and 1,000-grain weight (TGW) were quantified in 2006 and 2007.

A strong positive correlation was found between IIS or Log₁₀*Tri5* DNA and total DON concentration in the harvested grain. The Js399-19, tebuconazole, and the mixture of tebuconazole and thiram significantly reduced IIS of FHB, amount of *Tri5* DNA, and total DON within the grain and increased TGW. Although azoxystrobin, carbendazim, and thiram can increase TGW, they had no effect on the occurrence of *F. graminearum* compared with those of the untreated controls. Surprisingly, azoxystrobin and carbendazim significantly increased the total DON content in the harvested grain because they might have stimulated the amount of total DON production per *Tri5* DNA. The fungicides Js399-19, tebuconazole, and the mixture of tebuconazole and thiram were the most effective in controlling FHB and reducing DON contamination of the wheat.

Additional keyword: mycotoxins.

Fusarium head blight (FHB), also known as scab, is a destructive fungal disease of wheat and other small grain cereals throughout Europe (29), the United States (26), Canada (14), and China (4). Although 17 casual organisms have been associated with FHB (29), *Fusarium culmorum* and *F. graminearum* (teleomorph *Gibberella zeae* (Schwein.) Petch) are the species most commonly associated with the disease. In China, *F. graminearum* is the predominant species (21,29). In recent years, yield loss in small grain cereals due to FHB epidemics has been estimated to be in the order of 10 to 70% (25). FHB epidemics occurred frequently in the middle and downstream regions of the Yangtze River and in the northeastern region in the past. However, with field climate change for high-yield culture, it has spread to the main wheat-production areas of northern and western China in the last decade.

The impact of FHB includes reducing cereal yield and grain quality as well as contaminating kernels with mycotoxins produced by *Fusarium* spp. Mycotoxin contamination causes the most serious economic concern because it can induce acute or chronic toxic effects when consumed by humans or livestock. Deoxynivalenol (DON) is the predominant trichothecene found in cereal grains in Europe, North America, and China (31,37). As a result, several countries have adopted advisory limits to ensure minimum levels of DON in finished products intended for human

consumption. To safeguard human health, the People's Republic of China Ministry of Health advises a limit of 4% scabbed grains in wheat and less than 1 mg kg⁻¹ for finished flour products (36). Similar guidelines have also been set for the United States (1 mg kg⁻¹), European Union (0.5 mg kg⁻¹), Austria (0.5 mg kg⁻¹), and Canada (2 mg kg⁻¹) (26).

At present, there are two major approaches to reducing DON contamination of grain: (i) application of fungicides at anthesis to reduce FHB (25) and (ii) selective breeding of cultivars resistant to *Fusarium* pathogens (28). In the absence of high levels of resistance within commercial wheat cultivars, most attempts to control FHB rely upon fungicide application. The curative systemic fungicide carbendazim, which belongs to benzimidazole group, is known to have a broad biological activity, including on *Fusarium* spp. on wheat plants. In China, this fungicide has been the most effective compound used to control FHB since the 1970s. Unfortunately, *F. graminearum* has developed serious resistance to carbendazim with a site-specific mode of action (39), leading to inefficacy of this fungicide. Fungicides containing triazoles as active ingredients are the most effective plant protection agents against *Fusarium* spp. at present (27) and considered to be an ideal substitute for carbendazim in China (17,20). Nevertheless, resistance against triazoles was reported in *Venturia inaequalis* (22) and *Mycosphaerella graminicola* (35). Azoxystrobin, belonging to a relatively new group of fungicides, had been introduced for use on cereals in 1998. It is highly active against *Microdochium nivale* and saprophytic fungi (34) but has limited activity against *Fusarium* spp. (33). Moreover, a novel chemical, Js399-19 (2-cyano-3-amino-3-phenylacrylic acetate), was devel-

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oped and used in China recently for controlling FHB (23). However, the effect of this fungicide on controlling FHB was evaluated just on the incidence of infected spikelets (IIS). The effect of it on DON contamination in the grain has not been reported.

Usually, evaluation of the effects of fungicides on controlling FHB mostly relied on the method of quantifying FHB pathogens. Current methods of quantifying FHB pathogens include (i) IIS (19), (ii) measure of fungal ergosterol (13), and (iii) detection of *Tri5* DNA of *Fusarium* spp. by semiquantitative polymerase chain reaction (PCR) (9,12). The first and second techniques estimate pathogen populations directly based on the incidence of the FHB pathogens which were a complex of *Fusarium* spp. and, therefore, non-species specific. Moreover, the third technique, based on conventional PCR, used an end-point measurement to quantify the amount of target DNA. Recently, a technique based on real-time PCR enabled calculation of the amount of PCR product present in a sample at a point of reaction in which strict exponentiality of DNA amplification has been described (11). Quantification of trichothecene-producing *Fusarium* spp. within harvested grain could be used to determine whether fungicides were affecting the proportion of trichothecene-producing *Fusarium* spp. within the FHB complex and, hence, altering the relationship between FHB severity and the amount of DON in harvested grain.

Today, fungicides must be safe to the environment and humans before being authorized for use. Nevertheless, the benefits of such fungicide application are worth investigation in relation to their efficacy in preventing or reducing toxin synthesis under field conditions. The objectives of this study were to (i) develop a real-time PCR assay to quantify the amounts of *F. graminearum Tri5* DNA within harvested grain; (ii) evaluate the efficacy of fungicides on FHB, total DON content, and the yield of wheat artificially inoculated with the same isolate of *F. graminearum*; and (iii) determine whether there was a correlation between IIS, quantity of *Tri5* DNA, and total DON content.

MATERIALS AND METHODS

Fungal material. A single-spore culture of a strain ZF43, which was identified as wild-type of *F. graminearum*, isolated from a scabby wheat grain in Zhejiang Province of China, was maintained on potato-dextrose-agar slants or plates at 4°C and used as needed. The ZF43 strain was highly pathogenic to winter wheat and had reliable levels of DON production (data not shown). For wheat head inoculation, conidia of *F. graminearum* were produced from a culture grown in 4% mung bean broth (1). The spore suspension was obtained by removing hyphal fragments through filtration and adjusted to a final concentration of 1×10^5 spores ml⁻¹ of water using a hemacytometer (Arthur H. Thomas Company, Philadelphia).

Field trial. During the 2006 growing season, winter wheat cv. Nannong No. 9918 was grown according to normal agronomic practices on Jiangpu Farm (Experiment Farm of Nanjing Agricultural University). Six fungicide treatments plus a control treatment without spraying were randomized in blocks (2.5 by 4.0 m for each plot of each replicate) according to a randomized block design with four replicates for each treatment. Plants were sprayed with the fungicides so that the solution was applied at 75 ml/m² using a precision hand sprayer when plants were at ear emergence (Zadoks growth stage [ZGS] 59) (38). The fungicides and the doses used are described in Table 1. When at midanthesis ZGS 65 (7 days after fungicide application), wheat spikes were artificially inoculated with the strain ZF43. Inoculation was achieved by spraying the prepared conidial suspension onto spikes using a knapsack sprayer at a rate of 33 ml/m². Plots were mist irrigated for 21 days following inoculation to produce conditions conducive to FHB development (18). The above procedures were repeated in the same field in the growing season of 2007.

Visual disease assessment and yield determination. The IIS was determined visually by counting the number of infected spikelets per ear 3 weeks after inoculation. Fifty ears were randomly chosen and analyzed for each replicate of each fungicide treatment. Efficacy percentage was determined by applying Abbott's formula: (IIS of control – IIS of treated)/IIS of control × 100. The wheat in the trial plots was harvested when ripe (ZGS 92) by hand threshing to ensure effective collection of all grains. A homogeneous 500-g sample of grain at 13% moisture was taken from each plot and bulked to provide a 2-kg sample per treatment. Four 50-g subsamples of grain were randomly selected to determine the weight (g) of 1,000 grains (TGW) of each fungicide treatment.

DNA extraction. DNA was extracted from pure fungal cultures using a modification of the method of Doohan et al. (8). A 3-day-old culture of *F. graminearum* in potato dextrose broth (25°C, 160 rpm) was filtered through sterile muslin and freeze dried. The resultant mycelia (0.5 g) was frozen in liquid nitrogen and ground to fine powder in a mortar. Then, 1 ml of cetyltrimethylammonium bromide (CTAB) buffer (sorbitol, 25 g; CTAB, 8 g; sodium chloride, 87 g; polyvinylpyrrolidone, 10 g; EDTA, 8 g; sodium dodecyl sulfate, 10 g; and water, 1 liter) was added to 100 mg of the powder in a 2-ml Eppendorf tube, mixed, and incubated at 65°C for 2 h. Following incubation, a 0.8-ml aliquot of supernatant was removed and added to an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The contents of the tubes were mixed by gentle inversion for 1 min and then centrifuged (12,000 × g) for 15 min. A 0.65-ml aliquot of the aqueous phase was removed to a fresh tube containing 0.65 ml of 100% isopropanol. The contents of the tubes were mixed by gentle inversion for 1 min, and the tubes were incubated at 4°C for 30 min and then centrifuged (12,000 × g) for 15 min. The resulting DNA pellets were washed twice with 70% alcohol and then

TABLE 1. Effects of fungicides on the incidence of infected spikelets, amount of *Tri5* DNA, total deoxynivalenol (DON) content, and 1,000-grain weight (TGW) in the winter wheat (cv. Nannong no. 9918) inoculated at Zadoks growth stage (ZGS) 65 with a conidial suspension of *Fusarium graminearum*

Fungicide ^y	Application rate (g a.i. ha ⁻¹)	Experiment 1 (2006) ^z						Experiment 2 (2007) ^z					
		IIS	EIIS	ADNA	EADNA	TDON	TGW	IIS	EIIS	ADNA	EADNA	TDON	TGW
Unsprayed control		30.6 a	...	279.1 a	...	9.3 a	32.5 d	25.1 a	...	228.6 a	...	7.7 a	39.9 d
Thiram	600	25.7 b	16.2	228.9 b	18.0	6.3 b	35.5 cd	21.2 b	15.4	188.6 b	17.9	5.2 b	43.7 bc
Carbendazim	375	19.3 c	37.2	153.9 c	44.8	9.1 a	38.9 bc	17.2 c	31.4	138.1 c	40.6	8.1 a	43.5 c
Tebuconazole	125	7.7 d	74.7	71.6 d	74.3	1.3 c	44.7 a	7.1 e	71.8	64.9 e	73.5	1.2 c	46.3 ab
Azoxystrobin	250	16.8 c	45.2	135.6 c	51.4	9.6 a	39.8 b	13.8 d	45.0	110.6 d	53.0	7.8 a	44.7 abc
Js399-19	375	6.1 d	80.2	33.6 e	87.9	1.1 c	45.1 a	5.4 f	78.5	29.4 f	89.5	1.0 c	47.3 a
Tebuconazole + thiram	405	7.8 d	74.5	62.2 de	77.7	1.5 c	44.7 a	7.5 e	70.2	59.0 e	76.1	1.5 c	46.9 a

^y All fungicides were commercially available and sprayed at ear emergence (ZGS 59).

^z For statistical analysis, incidence of infected spikelets (IIS) was arcsine transformed and data were back-transformed means. The amount of *Tri5* DNA (ADNA) (µg kg⁻¹ of grain), total DON content (TDON) (mg kg⁻¹ of grain) and TGW (g) did not require transformation. Data followed by the same letter were not significantly different (df = 18; *P* > 0.05). Efficacy of fungicides on incidence of infected spikelets (EIIS) and amount of *Tri5* DNA (EADNA) values was means of four replicates.

air dried. Pellets were resuspended in 0.1 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 65°C for 1 h before storage at 4°C.

The method used for DNA extraction from grain samples was similar except that 20 ml of CTAB buffer together with 50 µl of proteinase K and 30 µl of RNAase were added to 1 g of grain fine powder in a 50-ml centrifuge tube. DNA from grain was diluted 500-fold in TE for use in a PCR amplification reaction. DNA content in 1 µl of DNA solution was equal to DNA content in 400 µg of grain.

Primer design. Primers for *Tri5* (primer Tr5F [AGCGACTA-CAGGCTTCCTC] and primer Tr5R [AAACCATCCAGTT-CTCCATCTG]), amplifying a 544-bp segment, were derived from conserved regions of *Tri5* in *F. graminearum* (10). They were synthesized by Sangon (Shanghai, China) and purified by high-performance liquid chromatography. Lyophilized primers were dissolved in water to a concentration of 10 µM and stored at -20°C.

Real-time quantitative PCR. PCR reactions were set up in 0.2-ml white opaque low-profile polypropylene tubes and covered with ultraclear caps (Bio-Rad Laboratories, Inc., Hercules, CA). Each tube contained 12 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA), 1 µl of internal passive reference dye (reactive oxygen species), 1 µl of primer mix (containing the forward and reverse primers, each at a concentration of 10 µM), and 1 µl of either water (negative control) or template DNA. Distilled water was added to give a total volume of 25 µl. Forty-two samples were run in ABI PRISM 7000 PCR (Applied Biosystems, Foster City, CA) using the following cycling protocol. Samples were preheated at 50°C for 2 min followed by 2 min at 95°C. Subsequently, 35 cycles of 15 s at 95°C, 30 s at 60°C, and 45 s at 72°C were run. Detection of fluorescent product was carried out after the last step of each cycle. Following the final amplification cycle, a melting curve was acquired by one cycle of heating at 95°C, cooling at 60°C, and slowly heating at 95°C with continuous measurement of fluorescence at 520 nm. The amount of *Tri5* DNA (ADNA) present in a sample was calculated from a standard curve using the ABI PRISM 7000 software package (Applied Biosystems). The unit of the amount of *Tri5* DNA present in grain sample was expressed as micrograms of *Tri5* DNA per kilogram of grain (µg kg⁻¹ of grain). Efficacy of fungicides on amount of *Tri5* DNA was determined by applying the formula (ADNA of control • ADNA of treated)/ADNA of *Tri5* DNA of control × 100.

The standard curve was produced using the following method. First, purified *F. graminearum* DNA solution was determined by absorbance at 260 nm and the concentration of purified *F. graminearum* DNA solution was calculated from a calibration curve (11). Second, dilution series of purified *F. graminearum* DNA (from the concentration of 100 to 0.001 ng/µl) was run on real-time PCR and threshold cycle (CT) values at which the amplification curves of each dilute solution crossing the crossing line were recorded. The crossing line was set manually to 19.60 and resulted in a standard curve with the lowest standard deviation. Third, the standard curve was generated by plotting these CT values as a function of logarithm values of the amount of fungal genomic DNA in series of dilution.

DON analysis. Trichothecene standards, including DON, 3-acetyl-deoxynivalenol (3-ADON), and 15-acetyl-deoxynivalenol (15-ADON), were purchased from Sigma (Sigma-Aldrich, Shanghai, China). The method of extraction, clean-up, and derivatization for trichothecene analysis was modified from Goswami and Kistler (15). A 200-g subsample of grain was randomly selected from 2 kg of sample per treatment and ground for 5 min in an all-purpose grinder (Tianjin Traditional Chinese Medicine Machinery Factory, Tianjin, China). A 20-g portion of ground fine sample was extracted for 1 h with 80 ml of acetonitrile + water (84 + 16, vol/vol) on a reciprocating shaker. A 4-ml extract was

eluted through a 1-g cleanup column containing C-18 + aluminum oxide (3 + 1, wt/wt). The elution was evaporated to dryness and derivatized with a trimethylsilylimidazole-rimethylchlorosilane mixture (100:1). The trimethylsilyl derivations of DON were identified and quantified using a Thermo Trace GC Ultra capillary column gas chromatograph equipped with an auto injector (AS3000), electron capture detector, and MN PermaBond SE-54-DF-0.25 (30 m by 0.25 mm interior diameter, film thickness 0.28 µm (Macherey-Nagel, Düren, Germany)). The temperatures of the injection port and the detector were 250 and 300°C, respectively. The column temperature was 80°C held for 1 min, 40°C min⁻¹ to 160°C held for 1 min, and then 30°C min⁻¹ to 280°C held for 5 min. The carrier gas was helium at flow rate of 1.08 ml min⁻¹. The experiment of toxin extraction and analysis was repeated three times.

Statistical analysis. All data were analyzed with the analysis of variance and means were separated with the Fisher's protected least significant difference test at *P* = 0.05 to determine whether fungicide had a significant effect on IIS, quantity of *Tri5* DNA, DON content, and TGW. Where data were not normally distributed, appropriate transformations were performed prior to analysis. Simple linear regression analysis was also performed in order to determine whether significant relationships existed between IIS, quantity of *Tri5* DNA, and DON content. All data analysis was carried out using the statistical software SAS GLM (SAS Institute Inc., Cary, NC).

RESULTS

Similar results were recorded for each fungicide treatment from the two experiments, although there was significant difference between the two sets of data (*P* < 0.001) (Table 1). Therefore, data from the two experiments are presented separately. Across all fungicide treatments, results obtained from experiment 2 revealed lower IIS, concentrations of *Tri5* DNA, and DON in harvested grain compared with results obtained from experiment 1. However, TGW of 2007 was greater than that of 2006. This may be explained by the difference in weather conditions when the two experiments were performed. The curve of air temperature during the trial in 2007 paralleled the curve in 2006 but the precipitation during the trial in 2007 was significantly lower compared with 2006. Therefore, weather in 2006 was more favorable to FBH disease development than in 2007 (Fig. 1).

Fungicide effects on IIS. All fungicides reduced significantly (*P* < 0.05) the IIS caused by *F. graminearum* compared with the untreated controls (Table 1). Of all fungicides tested, J3399-19, tebuconazole, and a mixture of tebuconazole and thiram at 1:4 provided the most effective control of FHB and was significantly (*P* < 0.05) better than all other treatments. However, statistical analysis revealed that there was a significant difference (*P* < 0.05) between J3399-19 and tebuconazole in experiment 2 (2007). Although applications of thiram, carbendazim, and azoxystrobin also significantly reduced IIS compared with the untreated controls, the percent control achieved was only 15.4 to 45.2%.

Fungicide effects on quantity of *Tri5* DNA. The standard curve achieved by plotting CT values against the logarithm values of the amount of fungal genomic DNA gave a linear relationship ($y = -2.58x + 20.73$, $r = 0.98$) over the range of DNA concentrations examined. The detection limit of the assay was determined to be 1 pg of genomic DNA per reaction. No signal was measured in the uncontaminated wheat sample used as negative control. All samples amplified at the recommended concentration (shown in DNA extraction method) resulted in CT values within the range of the purified *F. graminearum* DNA standards. The real-time PCR assay employed to quantify the trichothecene-producing gene *Tri5* revealed that concentrations of *Tri5* DNA in harvested grain were significantly (*P* < 0.05) lower when plants were treated with all fungicides compared with the untreated

controls (Table 1). The Js399-19 fungicide proved to be the most effective, providing 87.9% (experiment 1) and 89.5% (experiment 2) control on *Tri5* DNA in harvested grain. However the mixture of tebuconazole and thiram at 1:4 and tebuconazole alone were less effective on reducing *Tri5* DNA than Js399-19, with reductions in 2006 and 2007 of 76.1 and 77.7% and 73.5 and 74.3%, respectively. Of all fungicides, applications of thiram, carbendazim, or azoxystrobin were the least effective in decreasing *Tri5* DNA though they also significantly reduced *Tri5* DNA when compared with the untreated controls.

Fungicide effects on total DON, 3-ADON, and 15-ADON concentration in the grains. The gas chromatograph quantified the combined concentrations of DON, 3-ADON, and 15-ADON in harvested grain. Detection limits as assessed with pure reference substances at a signal-to-noise ratio of 3:1 were 25, 40, and 20 $\mu\text{g kg}^{-1}$ for DON, 3-ADON, and 15-ADON, respectively. No signal was measured in the uncontaminated wheat sample used as negative control. The amounts of total DON in the wheat inoculated with *F. graminearum* ranged from 1.0 to 9.6 mg kg^{-1} of grain. Js399-19, the mixture of tebuconazole and thiram at 1:4, tebuconazole, and thiram alone could significantly ($P < 0.05$) reduce the concentration of total DON compared with the untreated controls (Table 1), with reductions of 87.0 to 88.2%, 84.4 to 86.0%, 80.5 to 83.9%, and 32.2 to 32.5%, respectively. Statistical analysis revealed that there was not a significant difference ($P < 0.05$) between Js399-19, the mixture of tebuconazole and thiram, and tebuconazole. Applications of azoxystrobin and carbendazim had no significant effect on the DON concentration. In contrast, both azoxystrobin and carbendazim slightly increased the total DON content.

Fungicide effects on TGW. All fungicides significantly increased TGW in 2006 and 2007, except thiram, which did not raise the TGW significantly ($P < 0.05$) compared with the untreated controls in experiment 1 (2006) (Table 1). Of all fungicides tested, Js399-19, tebuconazole alone, and the mixture of tebuconazole and thiram at 1:4 provided the greatest increase of TGW (increasing TGW by 18.5 and 38.8, 17.5 and 37.5, and 16.0 and 37.5% for 2006 and 2007, respectively). Statistical analysis revealed no evidence of a significant difference between the enhancements of TGW achieved from treatments of Js399-19, tebuconazole, and the mixture of tebuconazole and thiram.

Relationship between IIS, quantity of *Tri5* DNA, and total DON content. Regression analysis revealed a significant ($P < 0.05$) linear relationship between IIS and total DON content or $\log_{10}$ *Tri5* DNA and total DON content in the grains (Fig. 2). For each fungicide, it was seen that an increase of IIS or $\log_{10}$ *Tri5* DNA resulted in concomitant increase in total DON content in the grains. However, the correlation coefficients between $\log_{10}$ *Tri5*

DNA and total DON content in seven fungicide treatments were higher than IIS and total DON content. In addition, linear equations ($y = ax + b$) between IIS or $\log_{10}$ *Tri5* DNA and total DON content in seven treatments shown that the slopes (a) for Js399-19, tebuconazole alone, and the mixture of tebuconazole and thiram were obviously lower than those for azoxystrobin, carbendazim, and unsprayed control.

DISCUSSION

Previous work had shown that incidence of infected spikelets of FHB and DON concentration in harvested grains may (3,16) or may not be closely correlated (12,24). We found a good and positive correlation between IIS of FHB and DON concentration in harvested grains. However, fungicidal control of FHB had proved inconsistent, and conflicting evidence existed regarding the effect of fungicides on the IIS of FHB and the mycotoxin accumulation in grain infected by *Fusarium* spp. In most cases, both FHB and DON content were reduced following fungicide application (3). However, in some instances, reduction of one variable was not accompanied by reduction of the other (3). In certain instances, fungicide treatments led to an increase DON levels in grain despite a reduction in disease severity (33). Results from this study showed that Js399-19, tebuconazole plus thiram, and tebuconazole alone were very effective on both IIS of FHB and DON concentration in harvested grains. However azoxystrobin was not effective on *F. graminearum* and resulted in a slight increase in total DON concentration in harvested grains. The results can be explained by the differences among azoxystrobin, tebuconazole, and Js399-19 in fungicidal activity against members of the fungal complex causing FHB. Azoxystrobin is known to be active against *M. nivale* (33), and tebuconazole and Js399-19 were shown to be very active against *F. graminearum* and other *Fusarium* spp. (23,33).

Similar to the instance of the IIS of FHB, Js399-19, tebuconazole plus thiram, and tebuconazole alone were very effective in decreasing the amount of *Tri5* DNA of *F. graminearum*. Linear regression analysis revealed a strong correlation between the biomass of *F. graminearum* and DON concentration in harvested grains. Such results were consistent with those from other researchers (9,12,32). Moreover we also found that the correlation coefficients between $\log_{10}$ *Tri5* DNA and total DON content in seven fungicide treatments were higher than those between IIS of FHB and total DON content. This may be due to the fact that the quantification of *F. graminearum* in harvested grain by *Tri5* DNA was more accurate than the IIS. In addition, the amount of *F. graminearum* in the harvested grain determined the amount of DON concentration in the same conditions. However, the linear

Fig. 1. Weather data (weekly average) for Jiangpu Farm of Nanjing Agricultural University during the trial. A, Precipitation and B, air temperature.

equations between IIS or $\log_{10}$ *Tri5* DNA and total DON content in seven treatments were different. The slopes for Js399-19, tebuconazole alone, and the mixture of tebuconazole and thiram were obviously lower than those for azoxystrobin, carbendazim, and unsprayed control. It seemed that fungicide Js399-19, tebuconazole alone, and the mixture of tebuconazole and thiram could reduce the efficiency of DON production per unit of pathogen, whereas azoxystrobin and carbendazim increased this efficiency. The results of applying tebuconazole were consistent with those reported in earlier studies (12,30), yet the results about azoxystrobin were also consistent with the ones from other studies (7,33). The results on the effect of Js399-19, carbendazim, and thiram on DON production are considered new findings. To prove whether fungicides can alter the efficiency of DON production per unit of pathogen, we can also determine the efficiency of

DON production per unit of *Tri5* mRNA in *F. graminearum* by fungicide treatments in vitro (6) or in vivo.

A number of researchers had reported that most fungicide treatments could raise the yield of wheat compared with untreated control. Results from field experiments in our study showed that all fungicide treatments increased the TGW. The results for tebuconazole and azoxystrobin were consistent with those from other researchers (2,16). However, of all tested fungicides, Js399-19 was the most effective in increasing TGW.

Results from the survey suggested that the excellent effect of carbendazim on FHB has been reduced, and the azoxystrobin fungicide had the least effect on *F. graminearum*. Moreover, azoxystrobin and carbendazim significantly increased DON contamination in harvested grains. Therefore, there is a challenge now to find alternative fungicides, and carbendazim and azoxy-

Fig. 2. Relationship between **A**, visual disease assessment (percent infected spikelets) and **B**, quantity of *Tri5* DNA ($\log_{10}$ *Tri5* DNA) and total deoxynivalenol (DON) content in harvested grain of winter wheat (cv. Nannong no. 9918) from field plots sprayed with thiram ($\blacksquare$), carbendazim ($\bullet$), azoxystrobin ($\circ$), tebuconazole ($\diamond$), Js399-19 ($\triangle$), or a mixture of tebuconazole + thiram ($\blacktriangle$) at Zadoks growth stage 59; $\square$ = unsprayed control plot.

strobil should not be used in China, where *F. graminearum* is the predominant species causing FHB. To date, Js399-19 has been shown to be the most effective fungicide, because it can effectively reduce the IIS of FHB, *Tri5* DNA, and total DON contamination in wheat. Furthermore, it can also raise the weight of grain. Therefore, the fungicide Js399-19 might replace carbendazim for controlling FHB in the future in China, particularly in the areas where a carbendazim-resistant pathogen population have been detected (23). However, similarly to carbendazim, the Js399-19 fungicide also might be problematic due to high resistance risk, because *F. graminearum* isolates can easily produce resistance to it in vitro (5). Therefore, a practical recommendation should be that Js399-19 be applied alternately with tebuconazole or mixed with other fungicides. These strategies can manage the resistance risk and sustain the usefulness of this new product for the control of FHB.

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