Research

Sensitivity of *Fusarium graminearum* to Metconazole and Tebuconazole Fungicides Before and After Widespread Use in Wheat in the United States

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Abstract

Fusarium head blight (FHB) caused primarily by *Fusarium graminearum*, is a major disease of wheat in the United States. FHB is managed in part by applications of demethylation inhibitor (DMI) triazole fungicides during anthesis. The objective of this study was to examine the sensitivity of U.S. populations of *F. graminearum* to the DMI triazole fungicides metconazole and tebuconazole. Isolates of *F. graminearum* collected from wheat between 1981 and 2014 were tested for fungicide sensitivity using mycelial growth assays to determine the effective concentration at which 50% of fungal growth was inhibited (EC_{50}). A total of 45 isolates were tested for metconazole sensitivity and 47 for sensitivity to tebuconazole. Isolates were analyzed in groups based on collection date. Groupings consisted of isolates collected prior to widespread fungicide use in wheat (designated as year 2000) or after fungicides became available for use in wheat. The mean EC_{50} for isolates collected prior to 2000 was 0.0240 μg/ml for metconazole and 0.1610 μg/ml for tebuconazole. For both fungicides, isolates collected between 2000 and 2014 had significantly higher ($P = 0.05$) mean EC_{50} values (mean $EC_{50} = 0.0405$ and 0.3311 μg/ml for metconazole and tebuconazole, respectively) compared with isolates collected prior to 2000. Isolate, year, and state of collection all affected the mean EC_{50} values of isolates collected between 2000 and 2014. A single isolate collected from Illinois in 2012 exhibited EC_{50} values of 0.1734 μg/ml for metconazole and 1.7339 μg/ml for tebuconazole, indicating reduced sensitivity compared with the mean EC_{50} of other isolates collected between 2000 and 2014. This study is the first step toward developing a fungicide sensitivity monitoring program for *F. graminearum* in the United States.

Keywords: Fusarium head blight, demethylation inhibitor, DMI, mycelial growth assays, fungicide sensitivity

Fusarium head blight (FHB), caused by the ascomycete *Fusarium graminearum* (Schwabe), is one of the most devastating diseases of wheat (*Triticum aestivum* L.) in the United States (McMullen et al. 1997, 2012). The fungus infects wheat heads primarily during anthesis and then germinates to produce hyphae that colonize all parts of the wheat spikelet. Infection of the spikelets interrupts grain fill and causes lightweight, shriveled, salmon-pink kernels to form in the wheat head (Sutton 1982). The fungus also produces several mycotoxins, including deoxynivalenol (DON), which further reduces grain quality (O'Donnell et al. 2000).

FHB management is achieved using integrated pest management practices that combine the use of moderately resistant wheat cultivars with inoculum-reducing cultural practices and fungicide applications (Dill-Macky and Jones 2000; Mesterházy 1995; Willyerd et al. 2012). The most effective fungicide active ingredients currently labeled for use on FHB are metconazole, tebuconazole, prothioconazole, and pydiflumetofen (Mesterházy et al. 2003; Paul et al. 2008; Saldago et al. 2018). All of these active ingredients, except pydiflumetofen, are in the sterol biosynthesis inhibitor class of fungicides and are demethylase inhibitors (DMI) (FRAC 2017). These are site-specific, partially systemic fungicides

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that are effective on a broad range of fungi (Kuck and Scheinpflug 1986). They inhibit the demethylation of the 14-C in lanosterol, a precursor to ergosterol, which plays an important role in the integrity of fungal cell walls. This is accomplished via an inhibition of cytochrome P450 sterol 14α-demethylase. The result is a buildup of sterol ergosterol precursors and free fatty acids, which in turn disrupts normal fungal growth (Köller 1992; Schnabel and Jones 2001; Siegel 1981).

Although the first DMI fungicide, triadimefon (Bayleton; Bayer CropScience) was released in 1973, no fungicides with this mode of action were officially registered for use on wheat in the United States until late 2006, when prothioconazole was officially approved for use on wheat (Kuck and Scheinpflug 1986; McMullen et al. 2012; Morton and Staub 2008; Russell 2005). However, in 1997, amid a devastating FHB epidemic, North Dakota was granted a Section 18 Crisis Exemption under the Federal Insecticide, Fungicide, and Rodenticide Act to use tebuconazole. Similar Section 18 exemptions were issued at least once between 1998 and 2008 in each of six other states for the use of tebuconazole, before it became officially registered for use on wheat in the spring of 2008 (McMullen et al. 2012; Russell 2005). Metconazole was first registered for use on wheat in the United States in the spring of 2008 (McMullen et al. 2012). Studies have since demonstrated the ability of metconazole and prothioconazole + tebuconazole to successfully reduce both FHB index and DON by as much as 50 and 45%, respectively (Paul et al. 2008).

Since their registration, these active ingredients have been used annually for FHB management in many wheat and barley producing regions in the United States. Such widespread, annual use increases the risk of fungicide resistance, which is concerning since DMI-triazole fungicides have been placed in a Fungicide Resistance Action Committee (FRAC) group with medium risk of resistance developing (Brent and Hollomon 2007; FRAC 2017). Reduced sensitivity toward these fungicides has been reported in the populations of several plant-pathogenic fungi in wheat and barley, including *Botrytis cinerea*, *Blumeria graminis* f. sp. *tritici*, *Colletotrichum cereale*, and *Rhynchosporium secalis* (Elad 1992; Kendall et al. 1993; Meyers et al. 2019; Wong and Midland 2007). Until recently, shifts in *F. graminearum* sensitivity to DMI triazole fungicides had only been reported in Europe and Asia (Klix et al. 2007; Yin et al. 2009), with the exception of an isolate of *F. graminearum* from New York that was reported as resistant to tebuconazole in 2014 (Spolti et al. 2014). Because farmers rely heavily on DMI triazole fungicides to manage FHB, it is important to determine if sensitivity to this fungicide class is changing in *F. graminearum* populations in the United States.

The objective of this study was to examine the sensitivity of U.S. populations of *F. graminearum* to metconazole and tebuconazole. This study is the first step toward developing a fungicide sensitivity monitoring program in the United States.

Collecting Isolates for Fungicide Sensitivity Assays

Isolates of *F. graminearum* were collected from multiple states across years (Table 1). Because true baseline isolates of *F. graminearum* that were collected prior to the registration of DMI-triazole fungicides in the United States are difficult to obtain, we solicited isolates from the stored collections of John Leslie, Kansas State University; Shaobin Zhong, North Dakota State University; and Fred Kolb and Carl Bradley, University of Illinois. Through these collections, we were able to obtain *F. graminearum* isolates collected prior to widespread fungicide use in wheat (designated as year 2000). Isolates collected between 2000 and 2014 were considered to have potentially been exposed to fungicides and were obtained by isolating from stored cultures or from FHB-symptomatic wheat glumes and seeds.

To ensure isolate viability before testing for fungicide sensitivity, wheat spikes were inoculated in the greenhouse with suspensions prepared from each isolate, and then *F. graminearum* was reisolated from infected tissue. Inoculum was prepared using liquid mung bean extract, and point inoculations were performed on wheat heads at Feekes 10.5.1 using 10 µl of 5,000 macroconidia/ml suspension of each isolate (Bai and Shaner 1996). After visible symptoms and signs were present, tissue was collected, and *F. graminearum* was isolated in the laboratory by placing infected tissue in a diluted bleach solution (10%) for 90 s, followed by a rinse in sterile water for 60 s. The surface-disinfected material was then air dried on sterile paper towels and placed in Petri dishes containing potato dextrose agar (PDA, Becton-Dickson, Franklin Lakes, NJ) amended with ampicillin (200 ppb) (VWR, Radnor, PA). Petri dishes were incubated in a growth chamber (AR22-L2, Percival Scientific, Perry, IA) for 3 to 5 days at 25°C under 12-h light and 12-h dark conditions. Approximately 25 µl of sterile water was added to the surface of a PDA plate with mycelial growth. The mycelial mat was scraped using a sterilized needle to create a suspension. The suspension containing mycelia and macroconidia was spread across a new PDA plate using a sterilized 10-mm loop and then incubated for 16 to 24 h at 25°C on the laboratory bench. After incubation, each plate was examined under a stereomicroscope (M165, Leica Microsystems, Wetzlar, Germany) for evidence of germinated *F. graminearum* macroconidia. Macroconidia were identified based on morphological characteristics designated by Leslie and Summerell (2006). If present, a single macroconidium was cut from the agar using a flame-sterilized insect pin and holder (BioQuip, Rancho Dominguez, CA) and placed inside a new Petri dish containing PDA. Cultures were allowed to grow under the conditions described above for 5 days and afterward were transferred to plates containing sterilized popcorn kernels, where they were incubated for 7 to 10 days and allowed to colonize the popcorn kernels. Colonized kernels were removed from the

TABLE 1
Year of collection, state of origin, and fungicide sensitivity of 13 *Fusarium graminearum* isolates collected prior to the year 2000 and subjected to in vitro sensitivity assays

			EC_{50} (µg/ml)[z]	
Isolate	Year	State	Metconazole	Tebuconazole
FG-1	1981	ND	0.0425	0.2748
FG-2	1993	ND	0.0220	–
FG-3	1998	VA	0.0475	–
FG-4	1998	VA	0.0119	–
FG-5	1999	IL	0.0085	0.0835
FG-6	1999	IL	0.0073	–
FG-7	1999	KS	0.0116	0.0362
FG-8	1999	KS	–	0.0449
FG-9	1999	KS	0.0588	0.5877
FG-10	1999	NY	0.0071	–
FG-11	1999	NY	–	0.0378
FG-12	1999	OH	–	0.0623
FG-13	1999	OH	0.0237	–

[z] Values are concentrations that inhibited radial fungal growth by 50%; dash (–) indicates this isolate was not tested for a given fungicide.

plate, dried overnight, and placed in cryotubes for long-term storage.

Forty-five isolates of *F. graminearum* from 14 states, with collection dates ranging from 1981 to 2014, were obtained for testing for metconazole sensitivity (Tables 1 and 2), and 47 isolates collected from 13 states during the same period were screened for tebuconazole sensitivity (Tables 1 and 2).

Determining Effective Concentration (EC_{50}) Values of *F. graminearum* to Metconazole and Tebuconazole

F. graminearum sensitivity to fungicide was determined by measuring fungal radial growth on fungicide-amended PDA. Fungicide stock solutions were created by dissolving technical-grade tebuconazole (97.4% active ingredient; Bayer Crop Science, Monheim am Rhein, Germany) and metconazole (98.8% active ingredient; BASF, Ludwigshafen, Germany) in acetone at a concentration of 50 mg/ml for tebuconazole and 500 ng/ml for metconazole. Serial dilutions (1:10) were performed in acetone and were added to PDA that had been autoclaved and cooled (below 60°C). The final fungicide concentrations in the media were 0.01, 0.1, 1, 10, and 100 µg/ml (plus a control of 1 ml of acetone only) for tebuconazole and 0.001, 0.01, 0.1, 1, and 10 µg/ml (plus a control of 1 ml of acetone only) for metconazole.

To determine sensitivity of *F. graminearum* isolates to metconazole and tebuconazole, a 5-mm-diameter mycelial plug was taken from the outer actively growing edge of a 5-day-old culture and placed growth side down onto fungicide-amended PDA at the listed concentrations. Two replicate plates of each concentration were used per isolate, and each isolate was tested at least two times. Each plate was wrapped twice with Parafilm (Bemis, Neenah, WI) and placed in an incubator set at 25°C in the dark. After 5 days, the radial growth on each plate was recorded by measuring the diameter of growth twice in a perpendicular pattern. The initial mycelial plug size was removed from the diameter measurements, and an average of the two measurements for each plate was obtained. Percent growth reduction was calculated by subtracting the average diameter of growth on plates divided by the average diameter of growth on the control plates (acetone only) from 100, and then multiplying that result by 100.

The fungicide concentration that effectively inhibited radial fungal growth by 50% (EC_{50}) of the non-fungicide-amended control was determined for each isolate and fungicide using the linear interpolation method in SAS version 9.4 (SAS, Cary, NC; Pasche et al. 2004). The experiment was arranged as a completely random design and was repeated at least twice per isolate and fungicide. Because of time and space constraints, isolates were

TABLE 2
Year of collection, state of origin, and fungicide sensitivity for 54 *Fusarium graminearum* isolates collected between 2000 and 2014 and subjected to in vitro sensitivity assays

			EC_{50} (µg/ml)[y]	
Isolate	Year	State	Metconazole	Tebuconazole
FG-14	2000	MT	0.0237 cd[z]	–
FG-15	2000	MT	0.0563 bcd	0.5629 cd
FG-16	2006	LA	–	0.0301 d
FG-17	2010	IL	–	0.0558 d
FG-18	2010	IL	–	0.0944 d
FG-19	2010	IL	0.0338 cd	0.3663 cd
FG-20	2010	IL	0.0621 bcd	0.6206 bcd
FG-21	2010	IL	0.0274 cd	0.2743 cd
FG-22	2010	IN	–	0.0516 d
FG-23	2010	IN	0.0407 cd	0.2754 cd
FG-24	2010	LA	–	0.1192 d
FG-25	2010	LA	0.0328 cd	–
FG-26	2011	IL	0.0437 cd	0.3485 cd
FG-27	2011	NY	0.0854 bc	0.8539 bc
FG-28	2011	NY	0.0207 d	0.2068 cd
FG-29	2011	NY	0.0415 cd	0.4146 cd
FG-30	2011	NY	0.0975 b	0.8452 bc
FG-31	2012	IL	–	0.0837 d
FG-32	2012	IL	–	0.3631 cd
FG-33	2012	IL	0.0184 d	0.1480 d
FG-34	2012	IL	0.0360 cd	–
FG-35	2012	IL	0.0494 cd	–
FG-36	2012	IL	0.1734 a	1.7339 a
FG-37	2012	IL	0.0659 bcd	0.6594 bcd
FG-38	2012	IL	0.0438 cd	0.4379 cd
FG-39	2012	IL	0.0407 cd	0.4068 cd
FG-40	2012	OH	0.0326 cd	–
FG-41	2013	AR	0.0412 cd	–
FG-42	2013	AR	–	0.1775 cd
FG-43	2013	IL	–	0.1107 d
FG-44	2013	IN	–	0.0871 d
FG-45	2013	IN	–	0.0372 d
FG-46	2013	IN	0.0199 d	–
FG-47	2013	IN	0.0112 d	–
FG-48	2014	DE	0.0096 d	0.3081 cd
FG-49	2014	DE	0.0194 d	0.1062 d
FG-50	2014	IL	0.0275 cd	0.2018 cd
FG-51	2014	IL	–	0.0623 d
FG-52	2014	IL	–	0.0378 d
FG-53	2014	IL	–	0.0772 d
FG-54	2014	IL	0.0161 d	–
FG-55	2014	IN	0.0215 d	–
FG-56	2014	MD	–	0.1015 d
FG-57	2014	MI	0.0320 cd	–
FG-58	2014	MI	0.0413 cd	–
FG-59	2014	MI	–	0.4988 cd
FG-60	2014	MI	0.0285 cd	–
FG-61	2014	NC	–	0.0395 d
FG-62	2014	NC	–	1.2847 ab
FG-63	2014	NC	0.0240 cd	0.2207 cd
FG-64	2014	NC	–	0.2979 cd
FG-65	2014	NC	0.0342 cd	0.2580 cd
FG-66	2014	NC	0.0264 cd	–
FG-67	2014	NC	0.0398 cd	–
FG-68	2014	OH	–	0.3861 cd

(*Continued*)

[y] Values are concentrations that inhibited radial fungal growth by 50%; dash (–) indicates this isolate was not tested for a given fungicide.

[z] Within a column, values with different letters are significantly different at the $P = 0.05$ level.

grouped and tested in batches of up to 15, including an internal control isolate, using previously described methods for batch trial testing (Wong and Wilcox 2000). This method was used to validate reproducibility of each of the trials conducted. Isolates and trials that did not meet reproducibility standards were dropped from the experiment.

After determining EC_{50} values for each isolate and fungicide, isolates were grouped into two categories, with isolates collected prior to 2000 (before widespread DMI use) constituting one group and isolates collected between 2000 to 2014 (after widespread DMI use) constituting the second group. Data were analyzed using PROC GLIMMIX in SAS version 9.4, testing the main effects of isolate and fungicide concentration. Further analyses on isolates collected between 2000 and 2014 tested fixed effects of isolate, state, and year and their interactions, with replicate as a random effect on EC_{50} using PROC GLIMMIX in SAS version 9.4. Mean separations were based on least square means test at the $P \leq 0.05$ level.

Fungicide Sensitivity of *F. graminearum* Isolates to Metconazole

EC_{50} values of metconazole for all isolates (1981 to 2014) ranged from 0.0071 to 0.1734 μg/ml, with a mean of 0.0369 μg/ml. When comparing means of isolates collected prior to 2000 and between 2000 and 2014, collection year significantly affected EC_{50} (Table 3). EC_{50} values of isolates collected prior to 2000 ranged from 0.0071 to 0.0588 μg/ml, with an overall mean of 0.0240 μg/ml (Table 1).

The range of metconazole EC_{50} values for isolates collected between 2000 and 2014 was 0.0096 to 0.1174 μg/ml, with a mean of 0.0405 μg/ml (Table 2, Fig. 1). Isolate, state, year, and their interactions all had a significant effect on the mean EC_{50} over this time period (Table 4). Isolate FG-36 (collected from Illinois in 2012) had a significantly higher mean EC_{50} than all other isolates (Table 2). Isolates FG-30, FG-27, FG-37, FG-20, and FG-15 also had significantly higher mean EC_{50} values than all other isolates except FG-36 (Table 2).

TABLE 3
Least square means test of mean fungicide concentration that effectively inhibited radial fungal growth by 50% (EC_{50}) for isolates collected prior to 2000 and from 2000 to 2014

	Mean EC_{50} (μg/ml)[z]		
Fungicide	Pre-2000	2000–2014	*P* > F
Metconazole	0.0240 b	0.0405 a	0.0031
Tebuconazole	0.1610 b	0.3311 a	0.0076

[z] Within a row, values with different letters are significantly different at the $P = 0.05$ level. *P* value associated with test of significant differences among isolates from different collection periods.

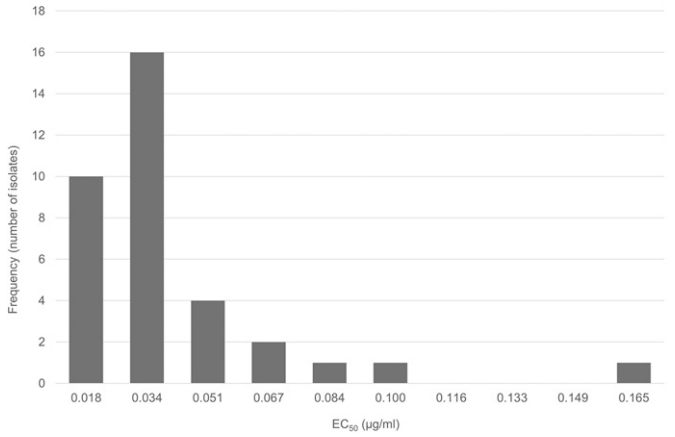

FIGURE 1
Frequency distributions of effective metconazole concentrations that inhibited mycelial growth by 50% (EC_{50}; μg/ml) for 35 *Fusarium graminearum* isolates collected between 2000 and 2014. Individual isolates are grouped in class intervals of 0.0164 μg/ml; values on the *x* axis are the midpoints of intervals.

Fungicide Sensitivity of *F. graminearum* Isolates to Tebuconazole

EC_{50} values for tebuconazole for all isolates (1999 to 2014) ranged from 0.0301 to 1.7339 μg/ml, with a mean of 0.3052 μg/ml. EC_{50} values of isolates collected prior to 2000 ranged from 0.0362 to 0.5877 μg/ml, with an overall mean of 0.1610 μg/ml (Table 1). When comparing means of isolates collected prior to 2000 and between 2000 and 2014, collection year had a significant effect on EC_{50} values (Table 3).

Mean EC_{50} values for isolates collected from 2000 to 2014 tested for sensitivity to tebuconazole ranged from 0.0301 to 1.7339 μg/ml with a mean of 0.3311 μg/ml (Table 2, Fig. 2). Isolate, state, year, and their interactions all had a significant effect on mean tebuconazole EC_{50} values (Table 4). Isolate FG-36 (collected in 2012 in Illinois) had a significantly higher EC_{50} than all other isolates collected from 2000 to 2014, except FG-62 (Table 2). The EC_{50} of isolate FG-62 was significantly higher than all isolates except FG-36 and FG-27 (Table 2).

Implications of *F. graminearum* Sensitivity to Tebuconazole and Metconazole on Future Research

Our study describes the first multistate screening of *F. graminearum* isolates for sensitivity to metconazole and tebuconazole in the United States. Although studies have been conducted showing a range of sensitivity in isolates across limited years and geographic areas to these fungicides (Spolti et al. 2012, 2014; Yin et al. 2009), this study is the first to show a significant difference in the EC_{50} values of isolates collected prior to 2000 from those collected from 2000 to 2014 to metconazole and tebuconazole. We were able to detect significant differences in EC_{50} values of isolates across states and years, indicating that isolates may be shifting in sensitivity due

TABLE 4
Estimates of fixed effects isolate, state, year, and their interactions on mean concentration of metconazole or tebuconazole that effectively inhibited radial fungal growth by 50% (EC_{50}, μg/ml) for isolates collected between 2000 and 2014

	Metconazole		Tebuconazole	
Fixed effects	F value	*P* value	F value	*P* value
Isolate	3.07	<0.0001	3.18	<0.0001
State	4.19	<0.0001	3.97	<0.0001
Year	7.36	<0.0001	7.41	<0.0001
Isolate × state	3.07	<0.0001	3.18	<0.0001
Isolate × year	3.07	<0.0001	3.18	<0.0001
Year × state	3.10	0.0003	3.21	<0.0001
Isolate × year × state	3.07	<0.0001	3.18	<0.0001

to more widespread use of DMI fungicides to manage FHB. Studies have shown that repeated exposure to DMI fungicides can reduce fungal sensitivity to these fungicides (Becher et al. 2010).

Our study indicates that there is variability in the range of *F. graminearum* sensitivity to metconazole and tebuconazole. Although our results indicate that *F. graminearum* sensitivity to metconazole and tebuconazole between 2000 and 2014 was on the whole lower than sensitivity of isolates collected prior to 2000, it should be kept in mind that these results are based on the small number of isolates available from before the year 2000. Access to isolates collected several decades ago is very limited because few isolates exist that were collected before widespread fungicide use. However, other studies have seen shifts in EC_{50} values with triazole fungicides and *F. graminearum* using small numbers of isolates (Avozani et al. 2014; Spolti et al. 2012, 2014; Yin et al. 2009).

In this study, no EC_{50} values above 2 μg/ml were observed for either tebuconazole or metconazole, and the range of values is consistent with reported ranges of metconazole (Spolti et al. 2014; Yin et al. 2009) and tebuconazole sensitivity distributions for *F. graminearum* (Spolti et al. 2014). Additionally, our findings were similar to previous reports (Klix et al. 2007; Spolti et al. 2014) that the EC_{50} values of isolates to metconazole were lower than the EC_{50} values of the same isolates to tebuconazole.

Previous work by Spolti et al. (2014) identified an isolate from New York (Gz448NY11) that was classified as resistant to tebuconazole. This isolate was also included in this experiment (FG-27; Table 2) and had a significantly higher EC_{50} value than many of the other isolates tested for both metconazole and tebuconazole but was not the isolate with the highest EC_{50} value reported. Differences in fungicide sensitivity observed between our study and Spolti et al. (2014) for isolates collected in New York and tested in both studies may be attributed to isolate availability and geographic distribution of isolates tested, and the use of formulated product in assays by Spolti et al. (2014) compared with technical-grade fungicides used in the current study. In Spolti et al. (2014), only isolates from New York were tested, whereas our analysis included isolates from other major wheat-producing regions of the United States where fungicide use is more prevalent. In our study, the isolate with the highest EC_{50} value for both metconazole and tebuconazole (FG-36) was collected in Illinois in 2012 from a research plot in Fayette County that had been treated with fungicide containing both tebuconazole and prothioconazole that year. The field where this isolate was collected served as a research location for rotated wheat fungicide trials and periodically received fungicide applications (C. Bradley, *personal communication*). A second isolate, FG-62, collected in North Carolina in 2014, had a similar EC_{50} value to FG-36 for tebuconazole but was not including in testing for metconazole. Interestingly, not all isolates tested from Illinois had significantly higher EC_{50} values compared with isolates from other states, and even isolates collected from other years in Illinois did not have high EC_{50} values. It appears that fungicide sensitivity may vary within fields as well as among fields or states, although a hierarchical sampling strategy would be necessary to confirm this hypothesis.

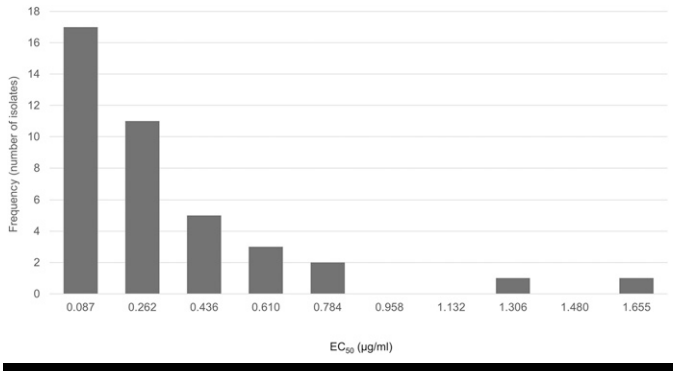

FIGURE 2

Frequency distributions of effective tebuconazole concentrations that inhibited mycelial growth by 50% (EC_{50}; μg/ml) for 40 *Fusarium graminearum* isolates collected between 2000 and 2014. Individual isolates are grouped in class intervals of 0.174 μg/ml; values on the *x* axis are the midpoints of intervals.

Although several different DMI fungicides are used in managing FHB in wheat, cross-resistance between specific fungicides has been shown in *F. graminearum* (Becher et al. 2010) as well as other fungal species (Avenot et al. 2016; Thomas et al. 2012). The consequence of this practice is that individual isolates may exhibit reduced sensitivity to several fungicides within the same chemical group even if an isolate has not been exposed to all fungicide active ingredients in that group. Fungicide exposure data for most of the isolates used in this study were unavailable; however, the cross-resistance between fungicides for specific isolates, such as FG-36, is important to note as new fungicides within the triazole group are developed for use on *F. graminearum*.

It is noteworthy that the isolate with the highest EC_{50} values in this study to both metconazole and tebuconazole was one that was exposed to triazole fungicides. Fungicide exposure data were not available for all tested isolates, making it impossible to quantify associations between fungicide exposure and EC_{50} values. The range in EC_{50} values reported from isolates across regions and years indicates that additional fungicide sensitivity assays are needed to test larger numbers of more recent isolates from a broad geographic range of wheat-producing areas in the United States.

Results from the analyses of data from FHB management field trials have shown that the fungicide metconazole and fungicides containing tebuconazole + prothioconazole are among the most effective at reducing FHB (Paul et al. 2008, 2010; Willyerd et al. 2012). Because the use of these three fungicide active ingredients is common in FHB management, determining the sensitivity of *F. graminearum* to all triazole fungicides is important. In our study, prothioconazole was initially tested in vitro in preliminary experiments using the same methodology as metconazole and tebuconazole. However, results were inconsistent, and few trials met the trial reproducibility standards outlined by Wong and Wilcox (2000). This is likely due to our use of technical-grade prothioconazole instead of the prothioconazole-desthio molecule, which is the molecule that conveys antifungal properties (Parker et al. 2013). Using prothioconazole instead of prothioconazole-desthio in in vitro fungicide sensitivity assays can still yield EC_{50} values for isolates tested; however, the inconsistency and reproducibility of these values is likely affected by the time needed for prothioconazole to break down into prothioconazole-desthio molecules. Although the time required for this conversion is documented, it has only been recorded in in vivo testing of plants and soil (Lin et al. 2017), and the rate of degradation in vitro is still unknown. Future studies involving in vitro testing of prothioconazole should consider using prothioconazole-desthio when designing and implementing fungicide sensitivity screening assays.

This research indicates that sensitivity of *F. graminearum* to metconazole and tebuconazole may be declining, indicating a need for comprehensive and widespread fungicide sensitivity monitoring of these and other fungicides applied for FHB management. Further studies should also examine the relationship between EC_{50} values and effectiveness of fungicide applications in vivo, examine the

impact of chemotype and its aggressiveness and ability to produce DON on fungicide sensitivity, and compare other fitness traits among isolates with different levels of fungicide sensitivity.

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