



Sensitivity to tebuconazole and carbendazim in *Fusarium graminearum* species complex populations causing wheat head blight in southern Brazil

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Abstract

Field trials where fungicides have been applied for *Fusarium* head blight (FHB) control were established and monitored in 17 municipalities in the Guarapuava region, Southern Paraná, Brazil, during four years (2011 to 2014). A total of 227 isolates belonging to the *Fusarium graminearum* species complex were obtained from symptomatic wheat heads. The majority of the 227 isolates (64%) were of the 15-(A)cetyl-deoxynivalenol (DON), 29% of the nivalenol (NIV) and 4% of the 3-ADON genotype. A random sample of 35 isolates was selected to determine the effective concentration of tebuconazole and carbendazim that reduces 50% of the mycelial growth (EC₅₀). The estimated means of the EC₅₀ ranged from 0.0004 to 3.0 and from 0.91 to 2.65 µg/ml for tebuconazole and carbendazim, respectively. Isolates of the 15-ADON genotype were less sensitive to tebuconazole compared to NIV + 3ADON. Evidence of lower sensitivity for both fungicides were detected. Discriminatory doses (carbendazim = 1.4 µg/ml and tebuconazole = 2.0 µg/ml) were used to screen the entire population (n = 227) for less-sensitive isolates. In the 2011–2014 population, four tebuconazole-less sensitive (2%) and nine carbendazim-less sensitive (4%) isolates were found, all originating from Guarapuava. A contemporary sample of isolates from 2018 to 2020 (n = 42) obtained from the same region were screened using the discriminatory doses. The frequency of the less-sensitive isolates increased over time from 2 and 4% to 24 and 95% for tebuconazole and carbendazim, respectively. Control efficacy was generally lower in plants inoculated with less-sensitive isolates. Greater number of less-sensitive individuals were recovered from plants inoculated with a mixture with a sensitive isolate and sprayed with either fungicide. The presence of less sensitive isolates suggest that selection may be associated with the frequent reports of decline in control efficacy for both fungicides tested in this study.

Keywords *Triticum aestivum* · *Gibberella zeae* · Chemical control · Fungicide resistance

Introduction

Fusarium head blight (FHB) is one of the most destructive diseases of wheat and small grains worldwide (McMullen et al. 2012). FHB epidemics may lead to significant losses in

crop yield (Duffeck et al. 2020) but also affect the quality of the grain due to mycotoxins that may accumulate at unsafe levels in the grains. The disease is caused by members of the *Fusarium graminearum* species complex (FGSC). At least five species have been reported associated with FHB in winter cereals in Brazil (Astolfi et al. 2011; Del Ponte et al. 2015). However, the dominant species (> 85%) in wheat in southern Brazil is *F. graminearum* 15-acetyl-deoxynivalenol (15ADON) genotype followed by *F. meridionale* nivalenol (NIV) genotype, which has a higher prevalence (around 30%) in the state of Paraná (Del Ponte et al. 2015). *F. graminearum* is a major DON producer, although some isolates are able to produce small amounts of its acetylated derivatives (3ADON, 15ADON), NIV and also zearalenone

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(Ward et al. 2008; Schmale et al. 2011; Del Ponte et al. 2012; Nicolli et al. 2015; Machado et al. 2022).

Despite the worldwide importance of the disease, FHB management is not an easy task. Although the disease is better managed integrating cultural practices, such as crop rotation to ensure residue management, and the use of moderately resistant cultivars, fungicide sprays play a major role to suppress disease development and mycotoxin accumulation (Wegulo et al. 2011).

Fusarium head blight (FHB) management heavily relies on the strategic use of fungicides, specifically when applied during peak flowering and alongside the cultivation of less vulnerable varieties, this strategy enhances the disease control (Mesterházy et al. 2003; Wegulo et al. 2011, 2015; Willyerd et al. 2012). Particularly, demethylation inhibitors (DMIs) have been persistently identified as superior in suppressing FHB and DON (Paul et al. 2008; Mesterházy et al. 2011, 2018; Machado et al. 2017). In the U.S. for example, metconazole or prothioconazole, either individually or combined, have exhibited superior efficacy, reducing FHB and DON more effectively than standalone uses of tebuconazole and propiconazole (Paul et al. 2008, 2018). In the context of Brazil, a meta-analysis demonstrated that tebuconazole outperforms propiconazole and carbendazim in terms of wheat yield, with an average increase of + 100 kg/ha (Machado et al. 2017) (Machado et al. 2017).

Quinone outside inhibitor (QoI) fungicides are typically not advised for Fusarium head blight (FHB) and deoxynivalenol (DON) management due to their reduced effectiveness relative to triazoles (Pirgozliev et al. 2003; Magan et al. 2011; Feksa et al. 2019; Bolanos-Carriel et al. 2020). In China, carbendazim, a benzimidazole chemical class, have been vastly applied but in recent years efficacy has decreased resulting in a failure of control (Liu et al. 2019). In fact, carbendazim-resistant isolates have been reported in China (Chen et al. 2007; Qiu and Shi 2014). In Brazil, carbendazim has also long use, prior to DMIs, for managing FHB (Deuner et al. 2011; Machado et al. 2017) which still remains as a cost-effective option for managing wheat diseases, including FHB, and little is known about fungicide resistance.

Information on sensitivity to triazole and even to benzimidazole fungicides among head blight pathogens infecting wheat in Brazil is scarce (Spolti et al. 2012a, b; Spolti and Ponte 2013; Avozani et al. 2014a, b) and it is still unclear whether there are any less sensitive isolates within the population. The present study is based on the hypothesis that there is resistance to carbendazim and/or to tebuconazole in FGSC populations in Brazil, especially in populations from regions with intensive application of fungicides. The objective of this study was to investigate the sensitivity to carbendazim and tebuconazole in a collection of *F. graminearum* associated with symptomatic wheat spikes from a high-fungicide-input region in south-central Paraná during

2011–2014. Additionally, we investigate the frequency of less-sensitive isolates in a sample from contemporary collection (2018–2020) obtained in the same growing region.

Material and methods

Study area and collection of isolates

Yearly survey for FHB symptoms was conducted on commercial fields of spring wheat at the Guarapuava region, southern Paraná, Brazil from 2011 to 2014. Symptomatic wheat spikes were collected at the field prior to kernel maturity. Grains were hand threshed, surface disinfected and placed on Petri dishes containing Nash-Snyder selective media (Leslie and Summerell 2006). After 7–10 days, isolates were obtained from colonies resembling *F. graminearum* sensu lato. Only one isolate was obtained from a single spike. Each isolate was purified from hyphae tips sub-culture and long-term preserved in SNA (*Spezieller Nährstoffmeragar*) (Astolfi et al. 2012; Gomes et al. 2015). Isolates were selected randomly from each year, and at least 21 or more isolates were obtained yearly, totaling 227 isolates.

A sub collection of *F. graminearum* 15ADON isolates obtained from symptomatic wheat heads in the same region in 2018 ($n=28$), 2019 ($n=3$) and 2020 ($n=11$) were also used in the discriminatory dose screening assay (Andrade et al. 2022).

Trichothecene genotypes identification

The potential to produce B-trichothecenes was assessed based on a multiplex PCR assay. Fungal mycelia was collected from 7-day-old *F. graminearum* cultures growing on PDA medium. Genomic DNA was extracted using CTAB protocol with some modifications (Astolfi et al. 2012; Gomes et al. 2015). Three B-trichothecene genotypes, 3-ADON, 15-ADON, or NIV, were determined based on the amplification of portions of Tri3 and Tri12 genes (Ward et al. 2002).

EC₅₀ and discriminatory doses determination

The EC₅₀ (effective concentration of the fungicide able to reduce 50% of the mycelial growth) of the fungicides tebuconazole and carbendazim were determined for a subsample of around 20% of the isolates ($n=35$), randomly chosen from the full collection. Aqueous stock solutions (100 µg a.i./ml) were obtained from commercially formulated tebuconazole (Folicur 200 EC; 20% of active ingredient [a.i.]; Bayer CropScience) and carbendazim (Carbomax 500 SC; 50% of a.i.; Nufarm Indústria química e farmacêutica S/A). Stock solutions of each fungicide were added to molten PDA

medium (45 to 55 °C). The tested concentrations for both fungicides were: 0 (non-amended agar—PDA), 0.5, 1.0, 2.0, and 4.0 µg a.i./ml. One mycelial agar plug (6 mm in diameter) from the edge of a 7-day-old-culture was placed in the center of a Petri dish (90 mm in diameter) containing 15 ml of PDA amended with fungicides at each concentration. Two mycelial plugs were placed equidistantly on plates at concentrations above 1.0 µg a.i./ml. Preliminary tests did not show any difference whenever one or two plugs were used (data not shown). Each plug was considered an observational unit, and two replicates were used for each concentration. After incubation at 25 °C for 4 days in the dark, radial growth was measured in two perpendicular directions using a digital caliper.

Screening based on discriminatory doses

The discriminatory dose for each fungicide was established as a single dose which allowed the separation of isolates into ‘sensitive’ or ‘less sensitive’ categories (Russell 2004). The entire collection of isolates ($n = 227$) were tested for both fungicides, tebuconazole and carbendazim, including the 35 isolates previously characterized. For such, one mycelial agar plug (6 mm in diameter) from the edge of a 7-day-old-culture was placed in the center of a Petri dish (60 mm in diameter) containing 7 ml of PDA amended with the discriminatory dose of 2.0 µg a.i./ml for tebuconazole and 1.4 µg a.i./ml for carbendazim. For each isolate, a non-amended agar plate was used as an untreated check. After 3 days of incubation at 25 °C in the dark, colony diameter was measured in two perpendicular directions using a digital caliper. Isolate was considered ‘sensitive’ whether its growth was lower than 50% at the discriminatory dose compared to growth on non-amended agar, and ‘less-sensitive’ otherwise (Russell 2004). The EC₅₀ assays, with the same range of concentrations as described previously, were performed for the less-sensitive isolates to confirm results using the discriminatory dose.

Molecular characterization of fungicide resistance

Partial sequences of *CYP51* and β_2 -*tub* genes of the two less sensitive (UEM 2536 and UEM 2657) and two sensitive (UEM 3587 and UEM 2795) isolates, were analyzed to detect mutations related to resistance to each fungicide. For such, isolates were grown in YES liquid medium (2 g of yeast extract, 15 g of sucrose for 100 ml of water) for 5 days at 25 °C. Mycelia were removed, dried in filter paper, and macerated in liquid nitrogen. Genomic DNA was extracted using cetyltrimethylammonium bromide (CTAB) protocol as previously described (Del Ponte et al. 2015).

PCR was performed in a final volume of 25 µL with 1 µL of DNA (~25 ng/µL), 1 µL of each primer at 10 µM, 16.9

µL of water e 2.5 µL Taq buffer, 0.5 µL of dNTP (0.4 mM each), 2.0 µL of MgCl₂ e 0.1 µL of Taq Polymerase (Kapa Biosystems). The *CYP51* primer sets F51A-F/R, F51B-F/R and F51C-F1/R1 were used to amplify the full sequence of *cyp51A*, *cyp51B* and *cyp51C* respectively (Yin et al. 2009). The primer Tub2seq-F/R was used to amplify the part of β_2 -*tub* gene (Liu et al. 2010). For *CYP51* primers, the amplification reaction consisted of an initial denaturation at 95 °C for 3 min, followed by 40 cycles at 94 °C for 1 min, 56 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The amplified fragment obtained has around 1200-pb. For β_2 -*tub* primers, the amplification reaction consisted of an initial denaturation of 95 °C for 3 min, followed by 35 cycles at 94 °C for 40 s, 57 °C for 40 s, 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. The amplified fragment obtained has around 1200-pb. Amplification was confirmed by using 3 µL of PCR product separated by electrophoresis on a 1% agarose gel. PCR products were purified using ExoSAP-IT (USB, Cleveland, OH) and sequenced by Macrogen Services (Kumchun-ku, Seoul, Korea) using each primer set used in PCR. Sequences were edited with DNA Baser Sequence Assembler v4 (Heracle BioSoft, 2013) and aligned using MEGA 7.0 (Tamura et al. 2013).

Fungicide efficacy and competitiveness assay under greenhouse conditions

Seed of ‘BR 18’, a spring wheat variety classified as highly susceptible to FHB, were sown in pots (10 seeds per 1-L pot) filled with substrate (Tropstrato®HT vegetables) fertilized with macronutrients (N-P-K at ratio 4–14–8). After the seedling emergence, plants were thinned to keep the 6 main tillers in each pot. Seedlings were fertilized weekly with 50 ml of nutrient solution containing 1.0 mM KNO₃, 0.25 mM NH₄H₂PO₄, 0.1 mM NH₄Cl, 0.5 mM MgSO₄·7H₂O, 1.0 mM Ca(NO₃)₂·4H₂O, 0.30 µM CuSO₄·5H₂O, 0.33 µM ZnSO₄·7H₂O, 11.5 µM H₃BO₃, 3.5 µM MnCl₂·4H₂O, 0.1 µM (NH₄)₆Mo₇O₂·4H₂O, 25 µM FeSO₄·7H₂O and 25 µM EDTA bisodium. Plants were kept in the greenhouse from planting to maturity.

Two factorial experiments were conducted spaced at 15 days between sowing dates. In the first experiment (3 × 5 factorial experiment), three treatments were tested (non-sprayed check, TEBU-sprayed, CARB-sprayed, and metconazole-sprayed) and five inoculation treatments consisting of isolates sprayed singly (S, R1 and R2) or in mixtures (S + R1, S + R2) were used. At early-milk stage, the plants were sprayed with tebuconazole (150 g a.i./ha, Folicur 200 EC; BAYER Corporation), carbendazim (250 g a.i./ha, Carbomax 500 SC, Nufarm Indústria química e farmacêutica S/A) or metconazole (81 g a.i./ha, Caramba 90 SL; BASF Corporation). Both fungicide-treated and nontreated plants (check treatment) were kept in the greenhouse for 48 h and spray inoculated with three

distinct inoculum compositions: the R1 (UEM 2536 isolate, less-sensitive to CARB and TEBU), R2 (UEM 2657 isolate less-sensitive to CARB and TEBU) and S (CML 3066, sensitive to CARB and TEBU, and highly aggressive on wheat) isolates inoculated individually or in mixture (1:1 ratio of S:R1 or S:R2). A macroconidia suspension (1×10^4 conidia/ml) was applied uniformly on wheat spikes using a household handheld sprayer at a rate of approximately 1 ml/head. Immediately after inoculation, plants were covered with a plastic bag and kept for 36 h in a growth chamber at 22 °C and a light–dark cycle of 12–12 h, respectively. The bags were removed and the plants remained in the greenhouse until harvest. Assessments of disease incidence and severity were made at 5, 10, and 15 days after inoculation in 6 spikes/pot in four pots (replicates) per treatment. The treatments were arranged in a completely randomized design with four replicates.

Before inoculation of wheat plants, less-sensitive isolates were evaluated for their ability to be distinguished from the sensitive isolate based on a vegetative compatibility assay (McCallum et al. 2004). For such, the absence or presence of barrage zones, when paired with their clonal cultures, were used to identify the recovered isolate from a dual inoculum treatment. At kernel maturity, a random sample of forty kernels, ten from each replicate of the S + R1 and S + R2 treatments, were obtained from each fungicide-sprayed and non-sprayed treatments. Kernels were incubated under wet conditions inside a plastic box containing a wet blotter paper. After incubating for three days at 25 °C with 12 h of light and darkness, *F. graminearum*-like colony developing on kernels provided a single isolate. For such, hyphae tips were subcultured on SNA plates and long-term preserved as described above. Four mycelial plugs of each recovered isolate were placed equidistantly around the outer edge of a Petri dish containing V8-wheat medium (150 ml of V8 juice, 20 g of ground wheat seed, and 20 g of agar per 1 L of medium) (McCallum et al. 2004). A mycelial plug of either inoculated isolate was placed at the center of the plates. After 10 days of incubation at 22 °C and continuous light, the presence of barrage zones was indicative of an incompatible reaction at the junctions of growing colonies, which allowed us to identify the recovered isolates.

Data analysis

The EC_{50} was calculated based on the linear regression between the relative mycelial growth inhibition (%) relative to unamend-check and the log-transformed fungicide concentrations (Becher et al. 2010; Liu et al. 2010; Spolti et al. 2014; Lehner et al. 2015). Plug diameter was previously subtracted from mean colony diameter. Multiple resistance was assessed based on the Spearman's rank correlation analysis ($\alpha = 0.05$) (because normality could not be assumed) for the relationship between EC_{50} values of tebuconazole and carbendazim. The distributions of EC_{50} values of each

fungicide, for each genotype, were compared using a Kolmogorov–Smirnov non-parametric test ($\alpha = 0.05$).

The area under disease severity progress curve (AUDPC) was calculated for each fungicide efficacy assay using function 'audpc()' from package 'agricolae' (De Mendiburu and Simon 2015). AUDPC data from each trial were tested individually for normality and homoscedasticity. Then, data were subjected to two-way analysis of variance (ANOVA). Fungicide spray, inoculum composition and trial (for the greenhouse experiment replications) were treated as fixed effects, and blocks (for the field trial) were included as a factor in our model to account for the effect of the blocking variable. When differences by the *F* test were significant ($P < 0.05$), the averages were compared by the Tukey test at 5% significance with the add-on package 'easynova' (Arnhold 2013).

In the competitiveness assay, the χ^2 test and Fisher's exact test (for small sample size) ($P > 0.05$) were used to evaluate the differences in the frequencies of recovered isolates from wheat heads inoculated with a mixture of sensitive and less-sensitive isolate. The frequency of the isolates was compared between carbendazim- or tebuconazole-sprayed and non-sprayed plants. All analyses and graphical work were performed using the R statistical computing environment (R Core Team 2021).

Results

Trichothecene genotypes frequency

In total, 227 isolates were obtained in 17 municipalities (Fig. 1A). The number of isolates ranged from 21 to 91 isolates among the years (Fig. 1B). The molecular assay based on *Tri3* and *Tri12* gene portions showed that the 15-ADON genotype was dominant overall (64%, 145/227), followed by the NIV genotype (29%, 65/227) and 3-ADON genotype (4%, 8/227). The molecular genotyping failed for eight isolates (4%, Fig. 1B).

EC_{50} determination

The EC_{50} values for tebuconazole ranged from 0.0004 to 3.15 $\mu\text{g/ml}$ and the overall mean was 0.39 $\mu\text{g/ml}$. For carbendazim, the EC_{50} values were 0.91 to 2.74 $\mu\text{g/ml}$ and the overall mean was 1.26 $\mu\text{g/ml}$ (Fig. 1C). Overall, the estimated EC_{50} values were lowest for tebuconazole and highest for carbendazim ($P < 0.005$, Table 1). There were three isolates with higher EC_{50} values for tebuconazole and one for carbendazim, all of the 15ADON genotype (Fig. 1C). The EC_{50} for tebuconazole and carbendazim varied among isolates of each trichothecene genotype group. However, the K-S test indicated that the sensitivity to tebuconazole of the 15ADON and NIV + 3ADON isolates were sampled from populations with different distributions, but not for carbendazim (Table 1).

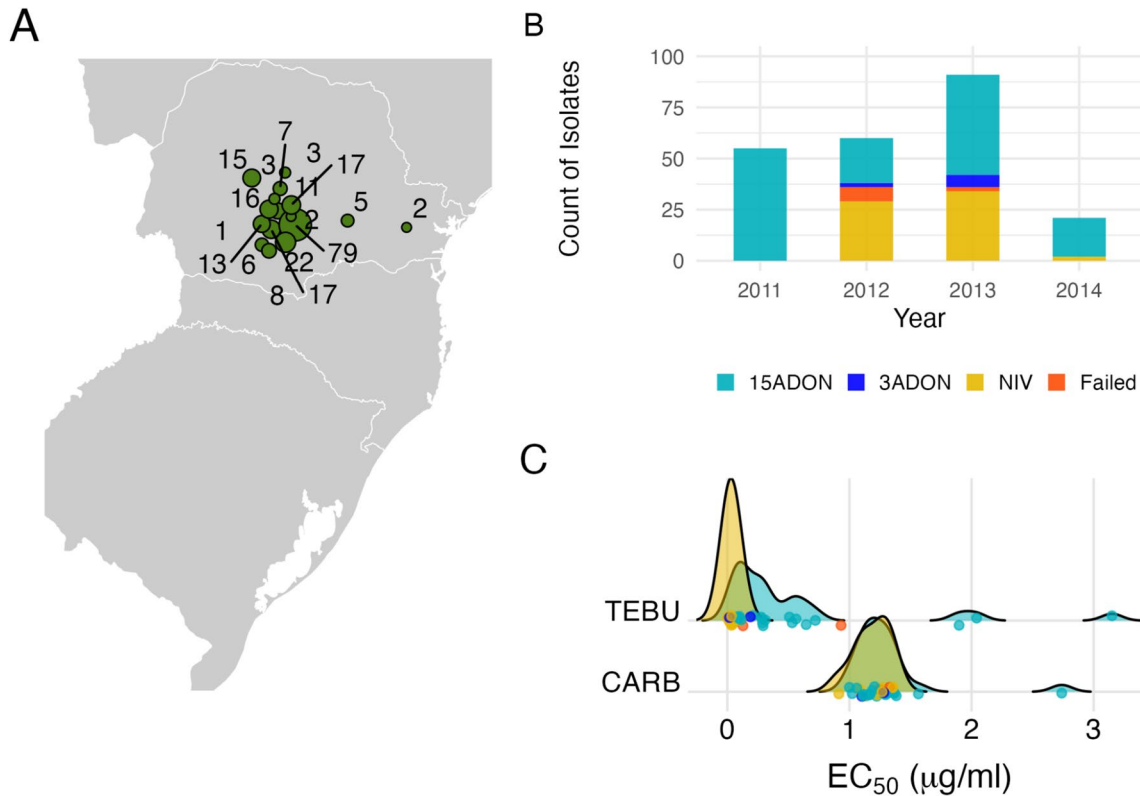


Fig. 1 A) Geographical location and number of *Fusarium graminearum* isolates ($n=227$) obtained from symptomatic spikes on high fungicide-input wheat fields across Paraná state in southern Brazil. B) Frequency of isolates per year characterized as 15-acetyl-deoxynivalenol (15ADON), 3ADON or nivalenol (NIV) trichothecene

genotypes. C) Density plots for the distribution of the effective concentration of tebuconazole (TEBU) and carbendazim (CARB) that reduces 50% of mycelial growth (EC_{50}) of a sample of 35 *F. graminearum* isolates, of 15ADON ($n=22$, blue density distribution) and 3ADON+NIV isolates ($n=11$, blue density distribution)

Table 1 Summary of carbendazim and tebuconazole sensitivity range and distribution of *Fusarium graminearum* isolates used in this study

Genotype	Fungicide	n	Mean	SE	Min	Median	Max	P -value ^a
15ADON	Carbendazim	22	1.29	0.07	1.00	1.21	2.74	0.63
NIV + 3ADON	Carbendazim	11	1.19	0.04	0.91	1.23	1.36	
15ADON	Tebuconazole	22	0.57	0.17	0.06	0.29	3.15	<0.05
NIV + 3ADON	Tebuconazole	11	0.05	0.02	0.00	0.03	0.19	
Overall	Carbendazim	33	1.26	0.05	0.91	1.22	2.74	<0.05
	Tebuconazole	33	0.39	0.11	0.00	0.10	3.15	

For each interaction, mean, standard error of the mean, minimum, median and maximum values were calculated from tested isolates (n) based on EC_{50} values ($\mu\text{g/ml}$)

^a P -value < 0.05 suggests that the sensitivity to tebuconazole or carbendazim of the group of isolates were sampled from populations with different distributions according to Kolmogorov–Smirnov non-parametric test ($\alpha < 0.05$)

Lower sensitivity between the two fungicides was detected based on the significance of the Spearman's correlation coefficient ($R = 0.30$, $P < 0.05$). In fact, the same isolate (UEM 2536) showed the highest EC_{50} for tebuconazole and carbendazim (Fig. 1C, Table 1).

Screening based on discriminatory doses

For tebuconazole, discriminatory dose was chosen based on outliers from the EC_{50} distribution of the 35 isolates (Fig. 2A). For carbendazim, the dose of 1.4 $\mu\text{g a.i./ml}$ used

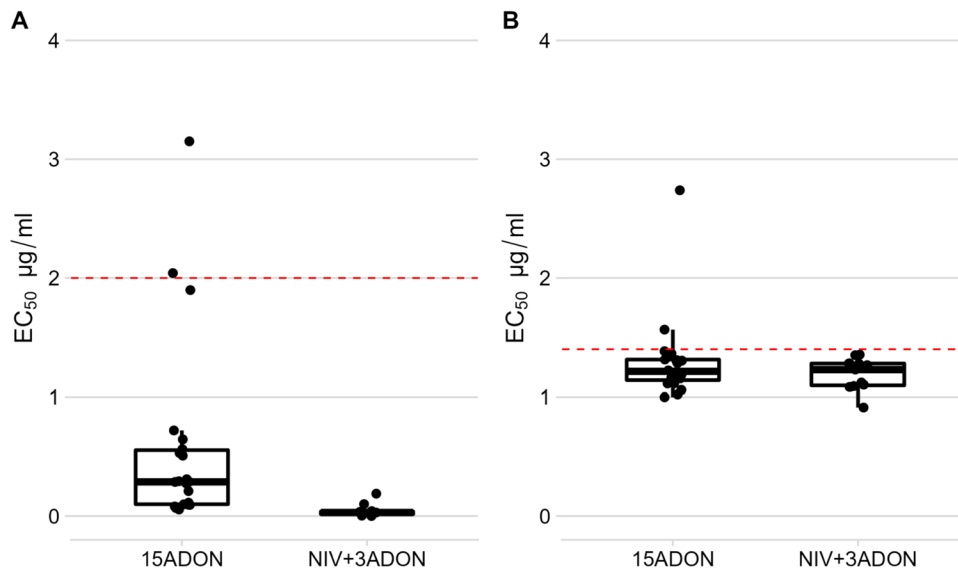


Fig. 2 Distribution of effective concentration of (A) tebuconazole (TEBU) and (B) carbendazim (CARB) that reduces 50% of mycelial growth (EC_{50}) for a sample of 33 *F. graminearum* isolates, of 15ADON ($n=22$) and 3ADON+NIV isolates ($n=11$). The horizontal line inside the box represents the median, the lines at the top and bottom of the boxes represent the 75% and 25% percentiles of the data,

respectively. The individual dots represent the mean value for each isolate. The points outside the 25% and 75% percentiles range represent outliers. Dashed red lines represent the defined discriminatory doses for tebuconazole (2 $\mu\text{g}/\text{ml}$) and for carbendazim (1.4 $\mu\text{g}/\text{ml}$)

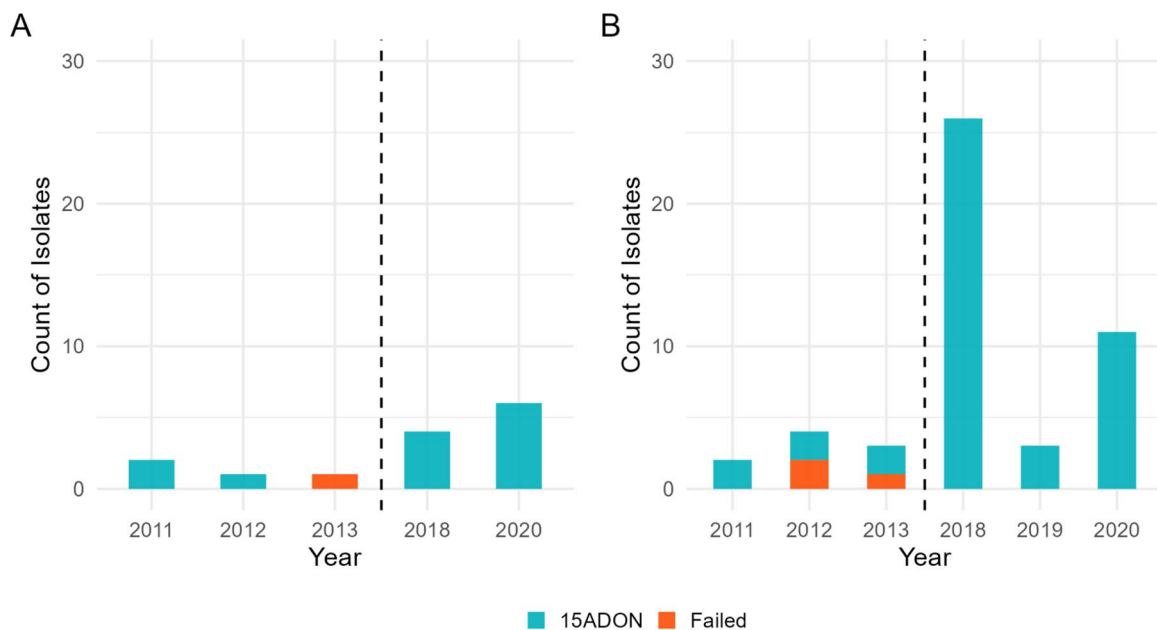


Fig. 3 Frequency of less sensitive isolates of *Fusarium graminearum* determined by the discriminatory doses of 2 $\mu\text{g}/\text{ml}$ for (A) tebuconazole and 1.4 $\mu\text{g}/\text{ml}$ for (B) carbendazim, across the 2011–2014 population ($n=227$ isolates, $n_{2011}=55$, $n_{2012}=60$, $n_{2013}=91$ and

$n_{2013}=21$) and 2018–2020 population ($n=42$, $n_{2018}=28$, $n_{2019}=3$ and $n_{2020}=11$). Isolate was considered ‘sensitive’ whether its growth was less than 50% at the discriminatory dose compared to growth on non-amended agar, and ‘less sensitive’ otherwise

in China (Liu et al. 2010) was initially taken as reference value, which was confirmed by the distribution of the EC_{50} values distribution of the subsample (Fig. 2B).

In the 2011–2014 population, four isolates were classified as less-sensitive to tebuconazole (2%, Fig. 3B), but only two were confirmed by the EC_{50} : UEM 2657 ($EC_{50}=2.44 \mu\text{g/ml}$) and UEM 2536 ($EC_{50}=3.00 \mu\text{g/ml}$). Nine isolates were considered less-sensitive to carbendazim (4%, Fig. 3B), and six were confirmed with the EC_{50} : UEM 2657 ($EC_{50}=1.56 \mu\text{g/ml}$), UEM 2655 ($EC_{50}=1.50 \mu\text{g/ml}$), UEM 3325 ($EC_{50}=1.61 \mu\text{g/ml}$), UEM 2519 ($EC_{50}=1.48 \mu\text{g/ml}$), UEM 2536 ($EC_{50}=2.65 \mu\text{g/ml}$) and UEM 3248 ($EC_{50}=1.56 \mu\text{g/ml}$). All of the less-sensitive isolates were obtained from plots in the municipality of Guarapuava. All less-sensitive isolates had the 15ADON trichothecene genotype, except UEM 2519 and UEM 3248 that did not have the genotype characterized (Fig. 3).

All but two isolates were considered less sensitive to carbendazim (95%) and ten isolates were classified as less-sensitive to tebuconazole (24%) in the 2018–2020 sample of isolates. An increase in frequency of less-sensitive isolates were observed for tebuconazole but more concerning for carbendazim.

Molecular characterization of fungicide resistance

Two selected isolates based on the response to carbendazim and tebuconazole discriminatory doses and confirmed by EC_{50} were sequenced to check for any mutation in target

genes. Analysis of the partial sequences of $\beta_2\text{-tub}$ and *CYP51* genes for the less sensitive isolates to carbendazim and tebuconazole did not identify any mutations compared with the reference sensitive isolate.

Fungicide efficacy

All the isolates tested caused FHB on wheat heads, but there was a large degree of variation in disease severity under greenhouse or field conditions. All data from the two greenhouse trials were pooled once there was no significant interaction between the data from the two trials and the other fixed effects ($P=0.401$). The interaction tested (fungicide spray vs isolates) did not affect the development of the disease ($P=0.843$). All the fungicides were able to reduce the AUDPC regardless of inoculum composition. Control efficacy, based on the AUDPC, was 25, 45 and 57% when plants were sprayed with carbendazim, metconazole and tebuconazole, respectively (Fig. 4A). Mean AUDPC did not differ between isolates and their mixtures, except for R1 causing the lowest amount of FHB (Figs. 4B).

Competitiveness assay

In total, 677 single-spored isolates were obtained from kernels inoculated with the mixture of isolates (69% the first trial and 31% from second trial). In total, 84, 14 and 2% of the recovered isolates were clonal to the sensitive highly-aggressive isolate (S) and the less-sensitive ones (R2 and

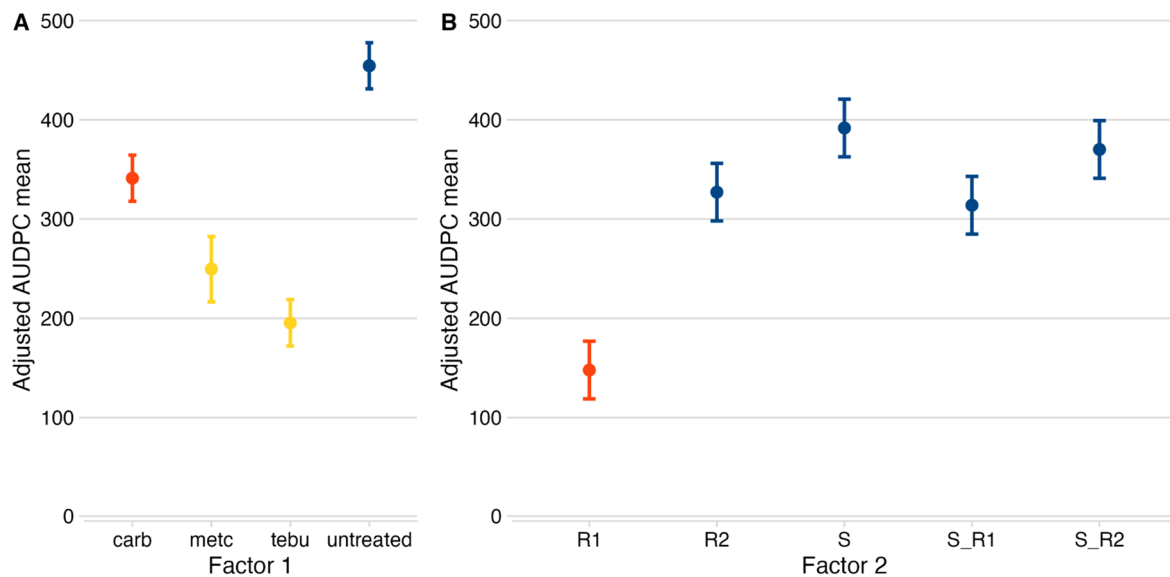


Fig. 4 Adjusted means and the respective standard error by the linear model fitted to the area under the disease severity progress curves (AUDPC) data pooled from the two greenhouse trials for Factor 1 (fungicide sprays) (A) and for Factor 2 (isolates) (B). Inoculum compositions: R1 (2536 isolate, less-sensitive to CARB and TEBU), R2

(2657 isolate less-sensitive to CARB and TEBU) and S (CML 3066, sensitive to CARB and TEBU, and highly aggressive on wheat) isolates inoculated individually or in mixture (1:1, S:R1 or S:R2). Means with different colors within each factor differed from each other based on Tukey test ($\alpha=0.05$)

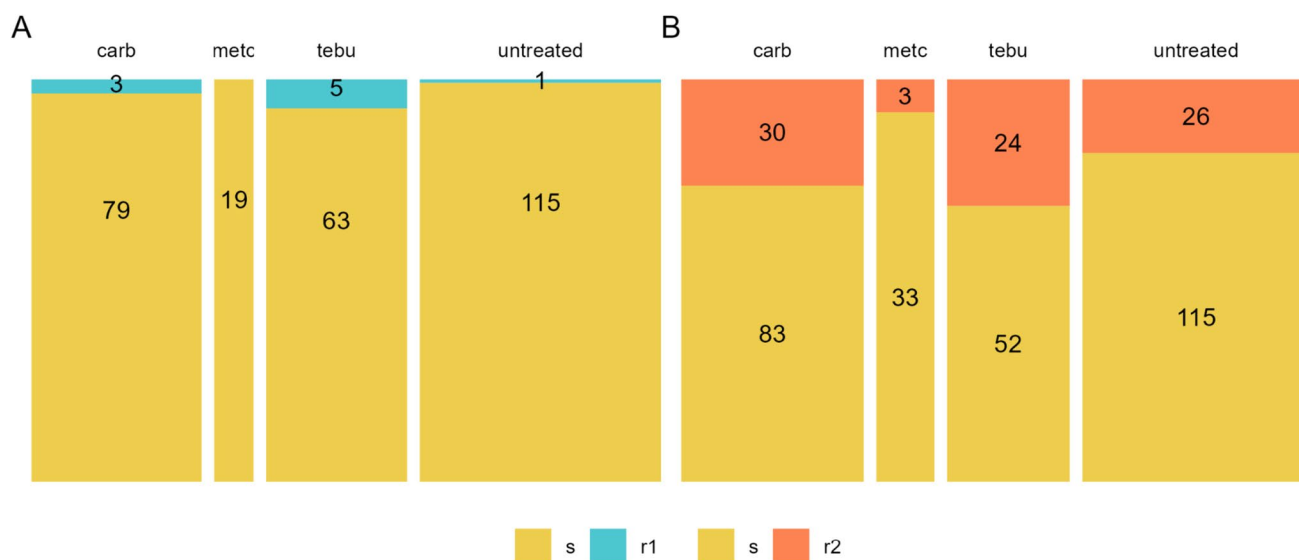


Fig. 5 Mosaic plots for the proportion of less-sensitive (r1, blue and r2, orange fill) and sensitive (s, yellow fill) isolates recovered from grains of wheat plants inoculated with an equal mixture of these isolates at early milk stage of grain development and sprayed with car-

bendazim (carb), metconazole (metc), tebuconazole (tebu) and water (untreated). Recovered isolates were identified based on the presence of barrage zones in incompatible pair matches (nonclonal)

R1), respectively. In non-fungicide treated plants, 82 and 99% of the recovered isolates were clonal to the sensitive isolate rather than R2 and R1, respectively. Grains from inoculated plants with the mixture of S + R1, an increase in frequency of the less sensitive isolate were observed when sprayed with tebuconazole ($P=0.027$) but not with carbendazim ($P=0.309$, Fig. 5A). Similarly, frequency of the less sensitive isolate R2 was higher in tebuconazole-sprayed plants ($P=0.041$) but not in carbendazim-sprayed plants ($P=0.130$) when compared with untreated plants (Fig. 5B). When metconazole was sprayed, there was no increase in the frequency of recovered less-sensitive isolates.

Discussion

This study provides the first characterization of the sensitivity to a DMI (tebuconazole) and a BMC (carbendazim) in isolates representing the most dominant FGSC species causing head blight in the south of Paraná State, Brazil. Although DMIs are most recommended for FHB control worldwide (Blandino et al. 2006; Willyerd et al. 2012; Chen et al. 2012), carbendazim has found use for FHB control in China for more than thirty years (Chen et al. 2007, 2012). There, several reports of carbendazim-resistant isolates have been made (Chen et al. 2007; Qiu et al. 2014). In Brazil, carbendazim has also long use, prior to DMIs, for managing FHB (Deuner et al. 2011) which has been a cost-effective option for managing wheat diseases, including FHB, and little is known about fungicide

resistance. Very recently, carbendazim-based products were banished to be commercialized in Brazil (ANVISA 2022), although growers can still use their own stocks. While previous study reported levels of sensitivity to two DMIs for FHB pathogen populations from southern Brazil (Spolti et al. 2012a, b), data on resistance to carbendazim are very limited. For instance, Avozani et al. (2014b) determined EC_{50} for carbendazim in only five FGSC isolates obtained from wheat, maize and barley from south of Brazil. The values ranged from 0.02 a 0.14 $\mu\text{g}/\text{mL}$. In our study, the mean EC_{50} for carbendazim was much greater (1.25 $\mu\text{g}/\text{ml}$), with six isolates being less sensitive to carbendazim (2.64%) based on a discriminatory dose determined for FGSC isolates from China.

Isolates exhibiting relatively high EC_{50} values for tebuconazole were reported in the United States (Spolti et al. 2014) and in China (Yin et al. 2009). In the state of New York, two least sensitive isolates showed EC_{50} of 8.09 $\mu\text{g}/\text{ml}$ and 2.46 $\mu\text{g}/\text{ml}$ in a collection of 50 isolates. In our study, only two isolates (0.8%) exhibited EC_{50} higher than 2.0 $\mu\text{g}/\text{ml}$. In Uruguay, the mean tebuconazole EC_{50} was 0.29 $\mu\text{g}/\text{ml}$ for *F. graminearum* ($n=42$) and 0.61 $\mu\text{g}/\text{ml}$ for *F. asiaticum* and *F. cortaderiae* ($n=14$) (Umpiérrez-Failache et al. 2013). The mean EC_{50} for the same fungicide in Rio Grande do Sul in a collection of 50 isolates was 0.0136 $\mu\text{g}/\text{ml}$ (Spolti et al. 2012b), much lower than the mean determine in the present study (0.39 $\mu\text{g}/\text{ml}$).

The increased mean EC_{50} and presence of less sensitive isolates in Paraná state compared with Rio Grande do Sul state may be due to the more intensive use of DMIs

for managing wheat diseases including FHB which may be selecting for fungicide resistance. Monitoring the sensitivity to fungicides as well as levels of efficacy in the field is critical, mainly for DMIs and MBCs. Thus far, declines in fungicide efficacy for FHB control over the years has not been reported, especially associated with fungicide resistance. In China, declines in the efficacy of carbendazim in the field has been linked to reduced sensitiveness of the pathogen to this fungicide (Chen et al. 2007).

Overall, 15-ADON isolates were less sensitive than NIV and 3-ADON isolates combined. In a previous study, *F. graminearum* isolates from Rio Grande do Sul, Brazil, were 20x less sensitive than *F. meridionale* isolates (Spolti et al. 2012b). In Uruguay, the mean EC50 for *F. asiaticum* and *F. cortaderiae* (0.61) were greater to *F. graminearum* (0.29 µg/ml, Umpiérrez-Failache et al. 2013). Although we did not identify the species, the 15-ADON isolates are probably *F. graminearum* according to a large survey conducted across years, locations and hosts (Del Ponte et al. 2015; Gomes et al. 2015; Kuhnem et al. 2016). However, the NIV has been assigned to more than one species, but mainly *F. meridionale*. Our results confirm the lower sensitivity of the dominant species causing FHB in Brazil (Spolti et al. 2012a, b; Nicolli et al. 2018), a fact that may help to explain its dominance over other species.

For the first time in Brazil, we report two isolates that were less sensitive to both tebuconazole and carbendazim, a fact that combined with the significant correlation between the EC50 values of both fungicides, suggest multiple resistance. The literature reports isolates resistant to tebuconazole and procloraz, an imidazole (Yin et al. 2009) as well as cross resistance between tebuconazole and metconazole (Spolti et al. 2012b).

We demonstrated that the frequency of less sensitive isolates to either carbendazim or tebuconazole increased over time specially in Guarapuava. This region is considered a high-fungicide input wheat producing region with at least three sprays per season to control FHB imposing a high selection pressure towards less sensitive isolates. Although the frequency of TEBU less-sensitive increases to 24%, a more concerning scenario is faced with CARB, where almost all isolates (95%) screened in a contemporary sample obtained on the same region were considered to have lower sensitivity.

Moreover, we demonstrated that the FHB control efficacy was generally lower in plants inoculated with less sensitive isolates under greenhouse and field conditions. In fact, a greater number of less-sensitive isolates were recovered from plants inoculated with a mixture of a sensitive highly-aggressive isolate and sprayed with either fungicide. These findings suggest that the divergence from the initial frequency of inoculated isolates may provide evidence of a selection pressure towards less-sensitive isolates in the

presence of either fungicide (Kretschmer et al. 2009; Spolti et al. 2014). This evidence corroborates with the increase of less-sensitive isolates obtained in a more recent sample of isolates. Conversely, the ratio of recovered isolates in the absence of either fungicide deviated from the expected 50:50% proportion, suggesting that it might have a pathogenic cost associated with fungicide less-sensitiveness trait of the tested isolates. Future studies should explore the adaptive cost for these isolates with low sensitivity in the absence of the fungicide, as well as whether a decline in control of *Fusarium* head blight might be associated with the increase of the frequency of less sensitive isolates under field conditions.

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Data availability The data and R codes used for the analysis of the data are available at: <https://doi.org/10.17605/OSF.IO/6AZSN>

Declarations

Conflict of interest The authors declare no competing interests.

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