Five-year Survey Uncovers Extensive Diversity and Temporal Fluctuations Among Fusarium Head Blight Pathogens of Wheat and Barley in Brazil

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Abstract

We conducted a five-year survey (2011–2015) of barley and wheat fields in Paraná state, Brazil, analyzing 754 *Fusarium* isolates from Fusarium head blight (FHB)-symptomatic spikes. Multilocus genotyping and TEF-1 α gene sequence analyses confirmed the dominance of the *F. graminearum* species complex (75.7%), but *F. poae* (11.5%) as well as *F. avenaceum* and related members of the *F. tricinctum* species complex (FTSC, 8.1%) appeared as substantial contributors to FHB. Within the FGSC, *F. graminearum* of the 15-ADON genotype was dominant (63%), followed by *F. meridionale* of the NIV genotype (23.1%), *F. cortaderiae* of the NIV (7%) or 3-ADON (2.6%) genotypes, and *F. austroamericanum* (3.8%) of the 3-ADON genotype. Substantial variation in pathogen composition was observed across years, with *F. poae* and *F. meridionale* frequencies significantly elevated in some years. Most *F. poae* strains produced DAS, diANIV and butenolide, but not Neosolaniol, T-2 or HT-2. All FTSC species produced moniliformin. Enniatin production was widespread among FTSC species, with the single *F. acuminatum* strain found to be the strongest producer of enniatins. Our findings confirm FGSC as a major contributor to FHB and expand considerably our knowledge of the presence, frequency and conditions under which other pathogens may emerge altering the spectrum of toxins that may accumulate in grain.

Introduction

Fusarium head blight (FHB) is a disease of major economic concern to wheat and barley growers due to the potential to reduce yield and contaminate grain with mycotoxins at levels that lead to grain downgrade or rejection by food and brewing industries (Goswami and Kistler, 2004; McMullen *et al.*, 2012). The disease manifests on the wheat heads as a result of infections by airborne spores of *Fusarium* spp. that land on the plant during flowering and early grain development stages (Del Ponte *et al.* 2007; Yoshida and Nakajima, 2010). Among several species of *Fusarium* that are pathogenic to these cereals, species of the *F. graminearum* species complex (FGSC) such as *F. graminearum* and *F. asiaticum*, are regarded as the main FHB pathogens worldwide, including in Brazil (Del Ponte *et al.*, 2015). The FGSC is a subset of the broader *F. sambucinum* species complex (FSAMSC), which includes species such as *F. poae* that are also commonly associated with FHB in some regions. In addition, *F. avenaceum* and other members of the *F. tricinctum* species complex (FTSC) are known to contribute to FHB epidemics, mainly in temperate regions (Gräfenhan *et al.*, 2013; O'Donnell *et al.*, 2013; Bec *et al.*, 2015).

The main concern with the presence of multiple toxigenic species relates to the wider range of mycotoxins that they may produce in grain. For instance, FGSC strains are known to produce B-trichothecenes, but subtypes can be determined for each strain given their ability to produce deoxynivalenol (DON) or nivalenol (NIV), and their respective acetylated forms 3-acetyl(A)DON (3-ADON chemotype), 15-ADON (15-ADON chemotype) and 4,15di-ANIV (NIV chemotype) (Savard *et al.*, 1991). Recently, a subpopulation of *F. graminearum* has been found to produce the type A trichothecene NX-2 (NX chemotype) instead of DON or NIV (Varga *et al.*, 2015). The genes responsible for the differential production of these toxins have been identified and assays have been developed to reliably

predict toxin type based on DNA sequence differences (Ward *et al.*, 2002; Liang *et al.*, 2014; Garmendia *et al.*, 2018). FGSC are also known to produce zearalenone, but differences among the species of the complex are still unknown. FTSC strains can produce moniliformin (MON) and a series of cyclic depsipeptides such as enniatin analogues (ENs) and beauvericin (BEA) (Jestoi *et al.*, 2008; Luz *et al.*, 2017). On the other hand, *F. poae* can produce NIV and type A trichothecenes in addition to BEA (Vanheule *et al.*, 2017).

In Brazil, molecular surveys since the mid-2000s have contributed to understanding FGSC diversity associated with FHB in wheat (Scoz *et al.*, 2009; Astolfi *et al.*, 2012; Del Ponte *et al.*, 2015) and barley (Astolfi *et al.*, 2011; Castañares *et al.*, 2016; Machado *et al.*, 2017). For those crops, *F. graminearum* is the dominant species from the FGSC (>80% frequency) along with four other DON or NIV-producing species within the complex. Expanding these molecular surveys to other major cereal crops documented host-specific differences in the relative prevalence of FGSC species. For instance, *F. meridionale* dominates in maize stalk and ear rots (Kuhnem *et al.*, 2015) and *F. asiaticum* dominates in rice kernels (Gomes *et al.*, 2015). Collectively, these studies suggest that FGSC composition and toxigenic profile are strongly shaped by host and regional factors. For example, *F. meridionale* was found to be a more significant contributor to FHB of wheat in Paraná state, where maize production is more common, than in Rio Grande do Sul (RS) state (Del Ponte *et al.*, 2015). In addition, there is evidence of a possible cultivar effect shaping the composition of FGSC species, as was demonstrated recently for barley grown in RS state (Machado *et al.*, 2017).

Previous studies of FHB in Brazil have largely focused on the diversity within the FGSC. Little is known about the presence and relative contributions of other species to FHB of wheat and barley in Brazil despite the potential significance of this variation for disease and mycotoxin control programs. In this study, we conducted a five-year survey of

FHB diversity in the central-south wheat-producing area of Paraná state, Brazil. This is one of the most important wheat and barley production regions in South America and was not well represented in previous studies. In addition to documenting the species and mycotoxin diversity associated with FHB in this region, the results were used to test the hypotheses that host or year of sampling drive pathogen composition.

Material and methods

Survey area, seasons and sampling

During five consecutive growing seasons (2011 to 2015), commercial wheat and barley fields from the central/south region of Paraná State Brazil were visited during the grain filling period and heads exhibiting FHB symptoms were manually harvested. The minimum and maximum distance between two fields was 26.2 km and 237 km, respectively. The fields were located within the latitudes of -24.45 to -26.05 and longitudes of -51.45 to -52.16 encompassing 17 municipalities. The wheat and barley fields were cultivated under no-till following soybean as summer crop.

Fungal isolation, purification and DNA extraction

All kernels were excised from each head and the *Fusarium*-damaged kernels selected for isolations. These were surface sterilized in 70% ethanol for 1 min, immersed in 1% sodium hypochlorite for 1 min, and rinsed three times with autoclaved distilled water. After drying, kernels were placed on top of wet filter paper inside a germination box and incubated for seven days at $20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ with a 12 hours light/dark cycle. Fragments of mycelia from a

typical *Fusarium* spp. colony grown on the kernels were transferred to water agar (WA) medium and sub-cultured onto a synthetic low-nutrient agar (SNA) using a single spore (Leslie and Summerell, 2006). Isolates were frozen at -80°C in 20% glycerol for long-term storage at the U. S. Department of Agriculture, Peoria, IL and at the Universidade Estadual de Maringá. A mycelial plug was transferred from the purified colonies to 6-cm petri plates containing V8 agar and incubated at 26°C for seven to 15 days. Mycelia were harvested with scalpel blades and stored at -20°C . Total genomic DNA was extracted using the Quick-DNATM Fungal/Bacterial 96 Kit (Zymo Research) according to manufacturer's instructions.

Species identification

Species and trichothecene genotypes were identified for FGSC isolates using a multilocus genotyping (MLGT) assay (Ward *et al.*, 2008). The MLGT is based on allele-specific primer extension (ASPE) reactions targeting single nucleotide polymorphisms (SNPs) for the discrimination of 16 species within the FGSC, five related species, and their associated trichothecene types (Ward *et al.*, 2008; Garmendia *et al.*, 2018). Sequences of the partial translation elongation factor (*TEF-1 α*) gene were obtained using previously published primers (O'Donnell *et al.*, 1998) for all isolates that failed identification in the MLGT assay. Polymerase chain reaction (PCR) was carried out in a final volume of 25 μL containing 50 ng of genomic DNA and 10x PCR Buffer, 50 mM MgSO_4 , 10 mM of deoxynucleoside triphosphate, 0.6 mM each primer, 5U of Platinum Taq High Fidelity polymerase (Invitrogen). PCR conditions were: initial denaturation for 2 min at 96°C followed by 35 cycles of 30 s at 94°C , 15 s at 53°C , 45 s at 68°C . PCR products were analyzed on a 1 % agarose gel and were purified with Millipore MultiScreen-PCR96 filter plates (Millipore).

Sequencing reactions were performed using Big Dye Terminator Sequencing kit v3.1 (Applied Biosystems) and analyzed with an ABI 3730 DNA Analyzer (Applied Biosystems). Sequences were edited and aligned manually using SeqAssem (Hepperle, 2004). Isolates were identified to the species level using sequence similarity searches of the Fusarium MLST (<http://www.cbs.knaw.nl/fusarium/>) and Fusarium ID (<http://isolate.fusariumdb.org/blast.php>) databases (Geiser *et al.*, 2004; O'Donnell *et al.*, 2010). Species identifications were applied when query sequences had at least 99% sequence similarity with reference sequences.

Analysis of species composition and frequency

Descriptive statistics summarized species composition and frequency between hosts and among years. The chi-square independence test (χ^2) or Fisher's exact test (for small sample size) were used to evaluate differences in species composition in specific comparisons. All data processing and analyses, as well as graphical work, were performed running R version 3.6.0 (2019-04-26) (R Core Team, 2019). To encourage and facilitate reproducibility, a website (<https://emdelponte.github.io/paper-Fusarium-PR/>) was generated to navigate through the documented code and all files are freely available and permanently stored as an Open Science Framework project available at <https://osf.io/dzc9h/>

Phylogenetic analysis of FTSC isolates

Phylogenetic analysis was conducted using TEF-1 α sequences from FTSC isolates identified in this study and 50 reference isolates (Niessen *et al.*, 2012; O'Donnell *et al.*, 2012;

Gräfenhan *et al.*, 2013; Lysøe *et al.*, 2014; Cerón-Bustamante *et al.*, 2018; Moreira *et al.*, 2019). DNA sequences were edited with SeqAssem (Hepperle, 2004) and aligned using the MUSCLE algorithm implemented in MEGA X (Kumar *et al.*, 2018). Phylogenetic analysis was also conducted in MEGA using maximum likelihood with the K2+G model of molecular evolution as determined by Bayesian Information Criterion (BIC) scores. Branch support was assessed via bootstrap analysis employing 1000 pseudoreplicates of the data.

Mycotoxin analysis

Isolates were screened for production of mycotoxins by inoculation on cracked maize kernels (25 g + 11 ml water) as previously described (Aoki *et al.* 2015). After 14 d incubation in the dark, 10 g of each culture was extracted with 20 ml 86:14 (v/v) acetonitrile:water for 30 min with shaking. After the extracts were clarified via filtration, they were analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS), using a ThermoDionex Ultimate U3000 liquid chromatograph (ThermoScientific) coupled to a Thermo Exactive high resolution mass spectrometer (ThermoScientific). A 0.6 ml/min reverse-phase gradient (40%–95%) flow between acetonitrile and water over 5 min was used to elute fungal metabolites from a 50 mm x 2 mm C18 column (Kinetex XB-C18, 2.6 µm particle size, 10 nm pore size; Phenomenex). HPLC-MS analyses were conducted in positive electrospray ionization mode to detect mycotoxins. HPLC-MS comparisons of ion mass and elution time with purified standards were used to identify the metabolites (Busman *et al.*, 2012; Busman, 2017). Limits of quantitation for aurofusarin, beauvericin, enniatin B, enniatin B1, enniatin A and enniatin A1 were 1 ng/µl.

Toxin production in liquid culture

F. poae strains were screened for trichothecenes, culmorin, and butenolide production in a liquid medium that induces trichothecene production. Each strain was initially grown on

V8 juice agar plates (20% V8 juice, 0.3% CaCO_3 , 2% agar; Stevens, 1974). Twenty ml of agmatine media (30 g sucrose, 1.14 g agmatine, 1 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g KCl, 10 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) and 200 μl of trace element solution (per 100 ml: 5 g citric acid, 5 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 50 mg H_3BO_3 , 50 mg $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$) per 1 L distilled water (Gardiner *et al.*, 2009) in 50 ml Erlenmeyer flasks were inoculated with two 0.5 cm^2 plugs cut from V8 plates. The cultures were grown at 28 °C on a rotary shaker at 200 rpm in the dark. After seven days each culture was transferred to a 50 ml conical tube and extracted with 8 ml ethyl acetate with shaking for 30 min. The mixture was separated with centrifugation (3000 rpm) and the top ethyl acetate layer was transferred to 1 dram vial and dried under a nitrogen stream. The residue was then re-suspended in 1 ml ethyl acetate for GC-MS.

GC-MS analyses were performed with an Agilent 5873 fitted with an HP-5MS column (30m, 0.25 mm, 0.25 μm), and detected with a mass spectrometer with an electron impact source. Helium was used as the carrier gas with a 20:1 split ratio and a 20 ml/min split flow. Samples were injected at 150 °C, the temperature held for 1 min and then the column was heated at 30 °C/min to 280 °C and then held for 7.7 min. Individual peaks in chromatograms were examined for trichothecenes. Under these conditions, T-2 toxin is detected at 10.6 min, neosolaniol at 8.5 min, 4,15-diANIV at 8.1 min, 4,15-DAS at 7.4 min, 15ADON at 7.1 min, and butenolide at 3.1 min. All chromatographic peaks were examined and identified based on comparison of retention time and ion fragmentation patterns with a commercial NIST library and a library prepared with purified standards. Extracted ion chromatograms were generated to screen for any components with a prominent m/z 121 ion, characteristic of T-2 toxin, neosolaniol and related compounds. The limit of detection was 0.25 μg toxin/ml culture.

Results

Species diversity

A total of 754 isolates of *Fusarium* spp. were obtained from symptomatic spikes. The number of isolates was similar between the hosts (Figure 1a), but variable across years; the lowest sample size was obtained from 2011 (n= 54) and the highest from 2014 (n = 295) (Figure 1b). Isolates were identified as members of nine species or species complexes (Figure 1c), among which FGSC accounted for the majority of isolates and occurred at similar frequencies between the hosts (79.9% and 72.4% of the wheat and barley strains, respectively). The second most prevalent species overall was *F. poae* recovered more frequently from barley (14.5%) than wheat (7.8%) followed by FTSC isolates found in similar frequency in wheat (7.5%) and barley (8.5%) (Figure 1c). Members of the FIESC and FFSC were represented each by around 2% of all isolates from both hosts. The isolates of *F. cerealis* were obtained from wheat and the single-isolate representatives of the *F. oxysporum* species complex (FOSC), *F. subtropicale*, and *F. armeniacum*, were obtained from barley (Figure 1a,2b). No significant relationship between the frequencies of the nine species/complexes and host (wheat or barley) (Fisher's exact test, $P = 0.071$).

Figure 1. Number of *Fusarium* spp. isolates in relation to host of origin (A) and cropping seasons (B) obtained from symptomatic heads sampled in wheat and barley fields sampled at several fields in the Guarapuava region, Paraná State Brazil, during five years. Isolates were identified as members of nine species or species complexes using DNA sequence-based methods (C)

Yearly frequency of *Fusarium* spp.

The frequencies of the species/complexes of the two host in combination, were dependent on the year (Fishser's exact test, $P < 0.001$) FGSC accounted for the majority of isolates in each of the five years; their frequencies ranged from 51.9 to 92.4% regardless of the host. *Fusarium poae* was present in all collections except wheat from 2001. Three distinct peaks

in frequency of *F. poae* were observed in isolates from wheat in 2013 (Figure 2a) and from barley in 2012 and 2013 (Figure 2b). A slight increase in FFSC was also observed in 2012 (Figure 2a,b). While FTSC was present in all years, FIESC occurred more sporadically but also peaked in 2012 in isolates from both hosts, similar to FFSC (Figure 2). The rare species (others) were collectively observed in four out of the five years.

Figure 2. Relative yearly frequencies of *Fusarium* species complex/species in a collection of 754 isolates obtained from heads of wheat (A) and barley (B) conditioned host of origin: FFSC = *F. fujikuroi* species complex; FGSC = *F. graminearum* species complex; FIESC = *F. incarnatum-equiseti* species complex and FTSC = *F. tricinctum* species complex; Others = *F. sub tropicale*, *F. armeniacum* and FO SC (*F. oxysporum* species complex)

FGSC composition and toxin genotypes

Four species were identified in the sub-collection of 571 FGSC isolates from both hosts. Among them, *F. graminearum* dominated the collection (63.3%) followed by *F. meridionale* (23.1%), *F. cortaderiae* (9.6%) and *F. austroamericanum* (3.8%) (data not shown). Differences in their relative frequencies in wheat and barley were not statistically significant ($\chi^2 = 7.40$; $P = 0.06$). For example, frequencies of *F. graminearum* were 58% and 69% in barley and wheat, and of *F. meridionale* were 26 and 19% in barley and wheat, respectively.

The four species were found in all years regardless of the difference in sample size. Their frequencies, wheat and barley combined, varied among the year (Fisher's exact test, $P < 0.001$). *Fusarium graminearum* was dominant in most year-host combinations, but its relative frequency was slightly reduced in 2012 and 2013, years when *F. meridionale* peaked in both hosts but was more evident in barley (Figure 3a,b). A slight increase of *F. meridionale* was also noted in 2015 in barley (Figure 3b). A single toxin type was observed for all species but *F. cortaderiae* isolates, which were either NIV (73%) or DON (27%). *F. graminearum* were all of the 15-ADON type, and all *F. meridionale* and *F. austroamericanum* isolates were of the NIV type.

FTSC diversity

Comparison of *TEF-1 α* sequences with reference sequences in the *Fusarium* MLST database suggested three FTSC species in the collection of 61 isolates. The results of phylogenetic analysis were consistent with the species identifications derived from *Fusarium* MLST. Three well-supported clades (Bootstrap $\geq 87\%$) were formed. The largest one included 51 *F. avenaceum*, 30 in wheat (Figure 4a) and 21 in barley (Figure 4b). The

second in frequency was FTSC11 with nine isolates (five from wheat and four from barley). *F. acuminatum* was represented by a single isolate collected in wheat in 2013. *F. avenaceum* was found in all but one season at frequency ranging from two to nine isolates and FTSC11 was found in all but two years in barley and all but one in wheat (Figure 4).

Figure 3. Number of isolates per species of the *Fusarium graminearum* species complex (FGSC) conditioned to year of sampling in wheat (A) and barley (B)

Mycotoxin production by *F. poae*

Three toxins were found in isolates of *F. poae* (20 from barley and 12 from wheat): 29/32 produced DAS, 24/38 produced diANIV and 20/32 produced butenolide (Table 1). Neosolaniol, T-2, or HT-2 were not detected in cultures of any of the isolates.

Figure 4. Number of isolates belonging to two clades of the *Fusarium tricinctum* species complex (FTSC) conditioned to year of sampling in wheat (A) and barley (B). A single *F. acuminatum* isolate was reported in wheat in 2013

Mycotoxin production by FTSC

Enniatins, moniliformin and beauvericin were detected but only the first two mycotoxins could be quantified. The frequency and concentration levels varied across the species.

F. avenaceum. All strains produced moniliformin, but not all produced the different forms of enniatins. Five out of 30 strains did not produce any form and 60% to 80% of the strains produced ENNA1, ENNB and ENNB1 at median levels ranging from 0.94 to 15.3; ENNA1 was detected at much lower concentration than ENNB and ENNB1 (Table 1).

FTSC11. Similar to the other species, all produced moniliformin. The frequency of enniatin-producing strains was similar to *F. avenaceum*, but the concentration levels were

much lower especially for ENNB and ENNB1. The maximum ENNB produced by one strain was similar to the median levels produced by *F. avenaceum* strains.

Table 1. Summary for the mycotoxin analysis data for 42 strains of the *Fusarium tricinctum* species complex (FTSC) isolated from Fusarium head blight-symptomatic heads collected at wheat and barley fields in southern Brazil (Guarapuava, Paraná state) during five growing seasons (2011 to 2015)

Species	Mycotoxin	Frequency	Mycotoxin concentration			
			Mean	Median	Min	Max
FTSC11	Enniatin A1	3	0.40	0.27	0.10	0.84
9 strains	Enniatin B	6	9.78	8.26	3.99	19.43
	Enniating B1	4	1.25	1.02	0.60	2.38
	Moniliformin	9	3.46	0.23	0.00	9.10
<i>F. acuminatum</i>	Enniatin A	1	7.76	-	-	-
1 strain	Enniatin A1	1	76.38	-	-	-
	Enniatin B	1	103.54	-	-	-
	Enniatin B1	1	116.30	-	-	-
	Moniliformin	1	3.05	-	-	-
<i>F. avenaceum</i>	Enniatin A	1	0.02	-	-	-
30 strains	Enniatin A1	20	3.57	0.94	0.15	22.46
	Enniatin B	24	28.59	15.28	0.16	106.81
	Enniatin B1	22	15.51	3.57	0.36	146.84
	Moniliformin	30	4.21	2.87	0.00	17.50

The only *F. acuminatum* strain produced moniliformin at a concentration similar to the mean concentration levels of the other two species. All forms of enniatin were produced by this strain, including ENNA, which was produced only this strain and one *F. avenaceum* strain (trace levels). Finally, this *F. acuminatum* strain produced very high amount of ENNA compared to the other two species ($>70 \times$ higher than the median) and amount of ENNB and ENNB1 similar to only two out of 30 *F. avenaceum* strains that produced these toxins at level greater than $100\times$.

Discussion

Results of this study expanded considerably our knowledge of the diversity of *Fusarium* species associated with FHB in wheat and barley in Brazil, in particular for a major small grain production region in South America. The Guarapuava region has not been well represented in previous surveys that identified FGSC isolates collected mainly from northern and western Paraná state (Del Ponte *et al.*, 2015). The analysis of the total FHB diversity obtained from a multi-season collection in a new region confirmed FGSC as the main FHB pathogen, but also revealed the presence of other non-FGSC species commonly known to cause FHB in other wheat-growing regions such as *F. avenaceum* and related FTSC members, as well as *F. poae*.

FTSC species are known to prevail in temperate, higher latitude, climates such as Northern Europe and Canada (Parry *et al.*, 1995; Bottalico and Perrone, 2002; Uhlig *et al.*, 2007). Studies conducted at lower, still cooler, latitudes have shown the presence of FTSC in wheat. In Kentucky, five in 68 strains from wheat belonged to two FTSC species (Bec *et*

al., 2015). In Mexico, 75% of 80 toxigenic isolates from wheat in the central highlands (cooler climate) belonged to *F. avenaceum* and a sister species (Cerón-Bustamante *et al.*, 2018). In Brazil, two previous studies have reported FTSC in wheat and barley. A small collection of FTSC strains was isolated from symptomatic wheat and air above canopy reproduced FHB symptoms in wheat at severity levels similar to FGSC (Moreira *et al.*, 2019). A recent survey also showed the presence of FTSC in commercial barley grain from southern Brazil (Piacentini *et al.*, 2019). The concern with an apparent emergence of FTSC among FHB pathogens relates to their toxigenic potential. In the Mexican study, most strains produced enniatin B or chlamydosporel. These species may potentially produce moniliform, which is known to cause muscular weakness, cardiovascular problems and reduced immune function, and reduced weight grain (Uhlir *et al.*, 2007). Our study showed that all FTSC strains, regardless of the species, were able to produce moniliformin. This is an important finding as data on grain contamination with this mycotoxin in Brazil is only available for maize as a results of *F. verticillioides* infection (Oliveira *et al.*, 2017). The *in planta* inoculation studies conducted in cooler regions where *F. avenaceum* is more common such as Canada and Poland, have shown the ability of this group to produce mycotoxin in barley and wheat grains (Goliński *et al.*, 1997; Abramson *et al.*, 2002)

Another known FHB-causing species reported for the first time at relatively high frequency in Brazil was *F. poae*, which represented 11% of the collection. *F. poae* is known to be a typical type B trichothecenes producer (NIV), but has also been known to produce type A trichothecene such as diacetoxyscirpenol (DAS) (O'Donnell *et al.*, 2018). In the Americas, *F. poae* isolates have been found in barley and wheat from Argentina and Canada (Bourdages *et al.*, 2006; Stenglein *et al.*, 2012), wheat in Paraguay (Arrúa *et al.*, 2019) and barley from Uruguay (Garmendia *et al.*, 2018). In Europe, reports exist in Germany, Italy and other countries (Birzele *et al.*, 2002; Xu *et al.*, 2005; Infantino *et al.*, 2012; Covarelli *et al.*,

2015). Identifications of *F. poae* strains based only on morphological characteristics are unreliable and may lead to inaccurate conclusions regarding the toxin production potential for this species. The species *F. langsethiae* is similar morphologically to *F. poae*, however it is more closely related to *F. sporotrichioides* than *F. poae* (Knutsen *et al.*, 2004; Torp and Nirenberg, 2004; Wilson *et al.*, 2004; Yli-Mattila *et al.*, 2004) and synthesizes the trichothecenes T-2 and HT-2, toxins not usually reported for *F. poae*. Therefore, DNA sequence-based identifications are required, and PCR assays may provide a rapid and reliable alternative to distinguish between these two species (Stenglein, 2009)

DON and NIV have multiple adverse effects at the molecular level, such as inhibition of proteins, DNA and RNA synthesis, with NIV representing an added concern for food safety, since several reports have shown its toxicity is higher than DON (Cheat *et al.*, 2015). The presence of NIN-producing *F. poae*, in addition to *F. meridionale*, as FHB pathogens in Brazil may represent an additional risk for wheat and barley, and could have negative effects on malting and brewing parameters (Nielsen *et al.*, 2014). Evidence of the large diversity of species in this region may also be explained by the occurrence of opportunistic pathogens at a relatively lower frequency.

Our data suggested yearly shifts in species frequencies, especially a decrease of the dominant *F. graminearum* population in favor of other contributors. In 2012, *F. poae* prevalence peaked in barley and *F. meridionale* peaked in both wheat and barley. The 2012 season was relatively drier and when the FHB incidence in the non-treated plots of experiments was generally low (Feksa *et al.*, 2019). We hypothesize that less-dominant pathogens may be more important in regions or years where weather is not favorable to *F. graminearum* outbreaks of FHB. In that context, understanding the overall spectrum of FHB pathogens and their mycotoxin potential may help inform toxin monitoring programs

when conditions are less favorable for *F. graminearum*. The ecological or seasonal factors driving these fluctuations should be further investigated, as this will have important implications for food safety.

When restricting the analysis to FGSC strains, our data confirmed i) the dominance of *F. graminearum* of the 15-ADON type, followed by *F. meridionale*, of the NIV type at a frequency similar to the other regions of Paraná state, and ii) the minor role of NIV-producing isolates of *F. austroamericanum* and *F. cortaderiae* in FHB epidemics. We confirmed the role of pathogen species in shaping the composition of the trichothecene genotypes in Brazil (Astolfi *et al.*, 2012; Del Ponte *et al.*, 2015; Gomes *et al.*, 2015; Kuhnem *et al.*, 2016). Despite analyzing close to two thousand isolates from different hosts, no chemotype polymorphism within *F. graminearum* from Brazil (exclusively 15-ADON) has been found. This is also the first study in Brazil as well as the first analyses of FHB pathogens from wheat in South America to screen for the novel NX toxin type, which has previously been reported among *F. graminearum* from North America (Liang *et al.*, 2014; Kelly *et al.*, 2015). As was previously reported in a smaller study of barley isolates from Uruguay (Garmendia *et al.*, 2018), the NX toxin type was not observed in Brazil.

The 3-ADON type in *F. graminearum* was reported for several strains in Argentina and for one isolate from Uruguay (Alvarez *et al.*, 2009; Umpiérrez-Failache *et al.*, 2013). In Brazil, the 3-ADON chemotype is unique to *F. cortaderiae* and *F. austromericanum* strains from wheat (Del Ponte *et al.*, 2015) and barley (Astolfi *et al.*, 2011; Castañares *et al.*, 2016). This pattern seems unique compared with other intensively sampled wheat regions of North America, Europe and China. There, the dominant species, either *F. graminearum* or *F. asiaticum*, possess more than one chemotype which alternate dominance depending on the region, previous crop or year. For example, 15-ADON and 3-ADON strains of *F.*

graminearum have been found in Europe where the former is more common in the south and central (Jennings *et al.*, 2004; Tóth *et al.*, 2005; Talas *et al.*, 2011; Boutigny *et al.*, 2014) and the latter in northwestern Europe (Yli-Mattila *et al.*, 2009; Nielsen *et al.*, 2012; Fredlund *et al.*, 2013). In China, *F. asiaticum* of the 3-ADON type has been replacing the NIV-producing populations of this species (Zhang *et al.*, 2012). In Japan, 3-ADON and NIV chemotypes from *F. asiaticum* predominate in wheat (Karugia *et al.*, 2009). In North America, 15-ADON chemotype is predominant in *F. graminearum*, but a high frequency of the 3-ADON chemotype isolates has been found in some parts of USA and Canada (Gale *et al.*, 2007; Ward *et al.*, 2008) and NIV and NX strains of *F. graminearum* have been reported at relatively low levels (Starkey *et al.* 2007; Gale *et al.* 2011; Schmale *et al.*, 2012; Kelly *et al.*, 2016). Finally, we found that NIV-producing FGSC species were more prevalent in barley than in wheat, which is in agreement with previous studies of isolates from these crops in RS state (Astolfi *et al.*, 2012; Del Ponte *et al.*, 2015; Machado *et al.*, 2017).

In summary, using state of the art methods of *Fusarium* species identification we report a much larger diversity of toxigenic species associated with FHB in wheat and barley in a major production region of southern Brazil. *F. graminearum* remains as the most dominant in wheat and barley, but FTSC and *F. poae* are reported here for the first time as significant contributors to FHB in Brazil and may be considered emergent pathogens. In addition, but at much lower levels, we report FFSC, FO SC, FIESC, *F. cerealis* and a new species within the FSAMSC, namely *F. subtropicale* (Pereira *et al.*, 2018). The significant temporal changes in FHB species composition could have direct impacts on the type of mycotoxins that accumulate in grain. Future mycotoxin surveys should focus on mycotoxins potentially produced by the entire spectrum of *Fusarium* species diversity

reported here. The continuous monitoring of the population may be informative to clarify the reasons of the temporal variation of the population in a certain year.

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