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Regional and field-specific differences in *Fusarium* species and mycotoxins associated with blighted North Carolina wheat

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ABSTRACT

Worldwide, while *Fusarium graminearum* is the main causal species of Fusarium head blight (FHB) in small-grain cereals, a diversity of FHB-causing species belonging to different species complexes has been found in most countries. In the U.S., FHB surveys have focused on the *Fusarium graminearum* species complex (FGSC) and the frequencies of 3-ADON, 15-ADON, and nivalenol (NIV) chemotypes. A large-scale survey was undertaken across the state of North Carolina in 2014 to explore the frequency and distribution of *F. graminearum* capable of producing NIV, which is not monitored at grain intake points. Symptomatic wheat spikes were sampled from 59 wheat fields in 24 counties located in three agronomic zones typical of several states east of the Appalachian Mountains: Piedmont, Coastal Plain, and Tidewater. Altogether, 2197 isolates were identified to species using DNA sequence-based methods. Surprisingly, although *F. graminearum* was the majority species detected, species in the *Fusarium tricinctum* species complex (FTSC) that produce "emerging mycotoxins" were frequent, and even dominant in some fields. The FTSC percentage was 50–100% in four fields, 30–49% in five fields, 20–29% in five fields, and $<$ 20% in the remaining 45 fields. FTSC species were at significantly higher frequency in the Coastal Plain than in the Piedmont or Tidewater ($P < .05$). Moniliformin concentrations in samples ranged from 0.0 to 38.7 μg g^{-1}. NIV producing isolates were rare statewide (2.2%), and never > 12% in a single field, indicating that routine testing for NIV is probably unnecessary. The patchy distribution of FTSC species in wheat crops demonstrated the need to investigate the potential importance of their mycotoxins and the factors that allow them to sometimes outcompete trichothecene producers. An increased sampling intensity of wheat fields led to the unexpected discovery of a minority FHB-causing population.

1. Introduction

Fusarium head blight (FHB) is a potentially devastating disease of small grain cereals worldwide. The disease is caused by several species in the genus *Fusarium* whose coexistence in the field is common (Ferrigo et al., 2016). The relative incidence and abundance of *Fusarium* species in wheat tissues may be dynamic across seasons (Köhl et al., 2007).

FHB-causing species are grouped into species complexes (Aoki et al., 2014; O'Donnell et al., 2013). Globally, most FHB is caused by members of the *Fusarium graminearum* species complex (FGSC) (Ferrigo et al., 2016; Starkey et al., 2007), which produce trichothecenes including deoxynivalenol (DON), nivalenol (NIV) and the recently discovered NX toxins (Varga et al., 2015). Members of the FGSC are in turn classified in the broader *Fusarium sambucinum* species complex (FSAMSC) along with other trichothecene producers such as *F. poae*, *F. sporotrichioides*, and *F. armeniacum* (O'Donnell et al., 2013).

Abbreviations: 15 acetyl-deoxynivalenol, (15AcDON); 3 acetyl-deoxynivalenol, (3AcDON); Beauvericin, (BEA); Confidence interval, (CI); Deoxynivalenol, (DON); Enniatins, (ENNs); Fusarium head blight, (FHB); *Fusarium fujikuroi* species complex, (FFSC); *Fusarium graminearum* species complex, (FGSC); *Fusarium incarnatum-equisetum* species complex, (FIESC); *Fusarium sambucinum* species complex, (FSAMSC); *Fusarium tricinctum* species complex, (FTSC); Gas chromatography- mass spectrometry, (GC-MS); Liquid chromatography- mass spectrometry, (LC-MS); Moniliformin, (MON); Multilocus genotyping, (MLGT); Nivalenol, (NIV); Translation elongation factor 1α, (*TEF1*)

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DON producers predominate in many countries, but NIV producers are more frequent in others. DON-producing *Fusarium* strains are further subdivided into 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) chemotypes based on differences in the trichothecene biosynthesis gene *TRI8* (Alexander et al., 2011). Outside North America, a north-south distinction has been observed in the European *F. graminearum* population, with 3-ADON isolates predominating in northern Europe and 15-ADON isolates predominating in central and southern Europe (Pasquali et al., 2016; Yli-Mattila et al., 2013). The majority of *F. graminearum* genotypes originating from small grains were of the 15-ADON chemotype in Germany, Austria, and portions of Russia (Yli-Mattila, 2010); the UK (Jennings et al., 2004); Denmark (Nielsen et al., 2012); Argentina (Reynoso et al., 2011); and southern Brazil (Astolfi et al., 2011). However, the 3-ADON type is common in Norway (Aamot et al., 2015), Poland (Stępień et al., 2008), Finland and northwestern Russia as well as the Russian Far East (Yli-Mattila et al., 2009), the middle and lower Yangtze River Valley of China (Zhang et al., 2010), and northern Japan (Suga et al., 2008). NIV producers were found to be dominant or very common in Iran (Haratian et al., 2008; Malihipour et al., 2012), Korea (Lee et al., 1986), western Japan (Nakajima and Yoshida, 2007), Nepal (Desjardins et al., 2004), and China's upper Yangtze Valley (Zhang et al., 2010).

In North America, 15-ADON has historically been the most common among the trichothecene chemotypes, but there are some regional differences. For example, the introduction of a novel genetic population led to a rapid increase in 3-ADON frequencies in some parts of Canada and the northern U.S. (Gale et al., 2007; Puri and Zhong, 2010; Ward et al., 2008). In Canada, trichothecene chemotype distributions and population dynamics among *F. graminearum* are characterized by two distinct longitudinal clines suggesting as yet unknown regional differences in the adaptive landscape (Kelly et al., 2015). In addition, *F. graminearum* that produce the novel type A trichothecene NX2 have recently been observed at relatively low frequencies in southern Canada and the northern U.S., and many of these strains belong to a novel population that may be endemic to this region (Kelly et al., 2016; Kelly and Ward, 2018; Liang et al., 2014). In a survey of FHB-causing isolates collected from various eastern states in the 1990s and 2000s, Louisiana yielded a high proportion of *F. graminearum* strains producing NIV or 3-ADON as well as NIV-producing *F. asiaticum* isolates, but only 15-ADON strains were found in the states of Illinois, Indiana, Kansas, Nebraska, and Ohio (Gale et al., 2011).

In the eastern U.S., a 2006 survey found low *F. graminearum* chemotype diversity in FHB-symptomatic spikes from 39 commercial wheat fields in New York, Pennsylvania, Maryland, Virginia, Kentucky, and North Carolina (Schmale et al., 2011). In New York, 3-ADON strains comprised 15% of isolates across all fields, and 45% in one field, while in the other states they were 0.5–8% of all isolates. In North Carolina, NIV strains were present as a minority (3%–22% of isolates) in three of the five fields sampled, but only one 3-ADON strain was detected out of 194 isolates genotyped (Schmale et al., 2011).

The relative frequencies of *Fusarium* trichothecene chemotypes in cereal fields are of practical importance. For example, in the USA, grain purchasers generally do not test loads of grain for NIV. There is evidence that NIV is more toxic to mammals than DON (Cheat et al., 2015; Minervini et al., 2004), while with respect to plants, Malihipour et al. (2012) suggested there is a gradient of aggressiveness, with 3-ADON producers being most aggressive to plants, followed by 15-ADON producers, and then NIV producers. In addition, trichothecene chemotype diversity among *F. graminearum* strains in North America has been a useful marker for genetic populations that differ in aggressiveness, growth and fitness characteristics, gene content, and other genomic features indicating they possess unique adaptations for use in exploiting the agroecosystem (Foroud et al., 2012; Kelly and Ward, 2018; Puri and Zhong, 2010; Spolti et al., 2014; Ward et al., 2008).

With respect to other FHB-causing species complexes, within the *Fusarium tricinctum* species complex (FTSC), *F. avenaceum* is the globally most important FHB-causing species, while *F. acuminatum* is also found sporadically (Aoki et al., 2014; Bottalico and Perrone, 2002). These species do not produce trichothecenes, and instead produce what have been referred to as “emerging mycotoxins,” in particular enniatins, moniliformin, and beauvericin (Beccari et al., 2019; Jestoi, 2008; Logrieco et al., 1998; Logrieco et al., 2002; Yli-Mattila et al., 2002). Outside North America, the FTSC has historically been important in FHB of cereals in northern Europe and Russia (Yli-Mattila, 2010). FTSC species were common in oat and barley grain samples from central and northwestern Russia (Stakheev et al., 2016); various cereals in Norway (Kosiak et al., 2003), Finland (Yli-Mattila et al., 2004), and Sweden (Lindblad et al., 2013); barley, durum and common wheat in France (Ioos et al., 2004); spring wheat in the Mexican highlands (Cerón-Bustamante et al., 2018); oats and barley from South Africa (Rabie et al., 1986); and in wheat and barley crops in New Zealand (Cromey et al., 2001).

It is not fully understood which factors determine the balance of *Fusarium* species of different complexes when all are competing in the same environment, although temperature and moisture are thought to be important influences that can favor one complex or another (Ferrigo et al., 2016). Within-season weather influences may play a role by affecting respective timing of spore liberation of competing species; for example, there is evidence that secondary metabolites from the more weakly pathogenic species *F. avenaceum* and *F. acuminatum* are favored when infections occur somewhat later in the anthesis period, as compared to *F. graminearum* which infects aggressively any time from 0 to 9 days after early anthesis (Beccari et al., 2019).

In North America, there have been only a few surveys of FHB-causing strains that examined in detail the incidence of non-FGSC species, and most were not in the U.S. Several Canadian surveys have detected a range of FSAMSC and FTSC species in grain. Among barley and wheat spikes grown in Prince Edward Island in 1982–1983, frequencies of 25% *F. graminearum*, 24% *F. poae*, 15% *F. avenaceum*, and 7% *F. culmorum* were found, with lower levels of *F. sporotrichoides*, *F. equiseti*, and *F. acuminatum* (Sturz and Johnston, 1985). A 1985 survey of Manitoba wheat found the majority of samples contained *F. graminearum*, *F. sporotrichioides*, and *F. equiseti*, while nearly half contained *F. poae*, *F. acuminatum*, and *F. avenaceum* (Abramson et al., 1987). In grain samples from three Canadian prairie provinces in the 1990s and 2010, *F. graminearum*, *F. culmorum*, and *F. avenaceum* were the most frequently isolated fusaria in rye and wheat samples, while *F. poae* and *F. sporotrichioides* were more commonly encountered in oat samples (Clear and Patrick, 2000; Gräfenhan et al., 2013). In samples collected from six Kentucky wheat fields, four spikes from one field yielded a total of two *F. graminearum* isolates and five FTSC isolates (three of which were *F. acuminatum* and *F. reticulatum*) (Bec et al., 2015). In southern Mexico, FSAMSC strains formed the majority in one state but FTSC strains predominated in an adjacent state (Cerón-Bustamante et al., 2018).

The aim of the present study was to intensively survey the FHB-causing population in North Carolina wheat, because previous surveys that included North Carolina FHB-causing isolates (Gale et al., 2011; Schmale et al., 2011; Walker et al., 2001) either involved very small samples or lacked the ability to distinguish members of the FGSC from other species. Moreover, as two of those studies detected the presence of NIV-producing strains in North Carolina, we wanted to determine whether NIV producers were distributed throughout the state, or were concentrated in higher frequencies in a specific zone. In this survey, we sought to deepen our understanding by intensively sampling FHB-causing isolates from a geographically diverse range of fields in the three basic small-grain cereal production environments of the southeastern Atlantic seaboard: the Piedmont, Coastal Plain, and Tidewater. We expected that *F. graminearum* would account for the entire sample or nearly so, and that there would be a small minority of NIV-producing strains. We were surprised to discover that in some fields, a large proportion or even a majority of strains isolated from FHB-symptomatic wheat spikes were non-trichothecene producers belonging to the FTSC.

Table 1
Percentages of four Fusarium head blight-causing species complexes in a total of 2197 *Fusarium* isolates collected from 59 wheat fields sampled in three agronomic zones of North Carolina in 2014.

Zone	Field	County	Nbr of isolates	Percent of sample[a]					
				FSAMSC	FTSC		FFSC	FIESC	NIV chemotype[c]
					%	CI[b]			
Piedmont									
	4	Union	22	81.8	13.6	3.8–38.5	4.6	0.0	0.0
	30	Rowan	18	61.1	5.6	0.7–33.3	27.8	5.6	0.0
	31	Rowan	47	93.6	0.0	–	4.3	2.1	2.1
	32	Davie	66	98.5	1.5	0.2–9.7	0.0	0.0	0.0
	33	Davie	19	63.2	36.8	17.4–61.8	0.0	0.0	0.0
	34	Davie	31	87.1	12.9	4.5–31.6	0.0	0.0	0.0
	35	Davie	18	94.4	5.6	0.8–29.7	0.0	0.0	0.0
	36	Davidson	61	98.4	0.0	–	1.6	0.0	0.0
	37	Davidson	24	95.8	4.2	0.6–23.6	0.0	0.0	0.0
	44	Wake	51	100.0	0.0	–	0.0	0.0	7.8
	45	Wake	25	88.0	8.0	1.7–29.9	4.0	0.0	0.0
	46	Stokes	13	61.5	30.8	10.5–62.9	7.7	0.0	0.0
	47	Forsyth	54	100.0	0.0	–	0.0	0.0	0.0
	48	Forsyth	43	95.4	0.0	–	4.7	0.0	4.7
	58	Cleveland	14	50.0	50.0	24.3–75.7	0.0	0.0	7.1
	59	Cleveland	9	67.0	33.3	10.5–68.1	0.0	0.0	0.0
Piedmont total			515	90.5	6.6	4.4–9.9	2.5	0.4	1.6
Coastal Plain									
	1	Pitt	54	98.2	0.0	–	1.9	0.0	1.9
	5	Lenoir	50	96.0	2.0	0.3–13.6	0.0	2.0	0.0
	6	Lenoir	20	95.0	0.0	–	0.0	5.0	0.0
	7	Lenoir	28	82.1	10.7	2.9–32.9	3.6	3.6	3.6
	11	Columbus	35	97.1	0.0	–	0.0	2.9	0.0
	12	Columbus	43	100.0	0.0	–	0.0	0.0	4.7
	13	Columbus	34	94.1	5.9	1.4–21.7	0.0	0.0	2.9
	14	Bladen	41	90.2	2.4	0.3–16.2	7.3	0.0	12.2
	15	Bladen	65	84.6	7.7	2.8–19.4	7.7	0.0	7.7
	16	Sampson	34	97.1	2.9	0.4–17.6	0.0	0.0	0.0
	17	Sampson	33	90.9	9.1	2.7–26.3	0.0	0.0	6.1
	18	Sampson	44	97.7	0.0	–	2.3	0.0	2.3
	19	Robeson	24	91.7	8.3	2.0–29.1	0.0	0.0	4.2
	20	Robeson	33	93.9	0.0	–	6.1	0.0	3.0
	21	Robeson	25	72.0	24.0	9.8–47.9	4.0	0.0	4.0
	22	Wayne	24	70.8	29.2	13.5–52.0	0.0	0.0	0.0
	23	Johnston	36	75.0	25.0	12.6–43.5	0.0	0.0	0.0
	24	Wayne	15	93.3	6.7	1.0–34.1	0.0	0.0	0.0
	25	Harnett	40	67.5	32.5	18.7–50.2	0.0	0.0	0.0
	26	Harnett	17	41.2	58.8	33.2–80.4	0.0	0.0	5.9
	27	Johnston	19	36.8	63.2	38.2–82.6	0.0	0.0	0.0
	28	Johnston	43	86.1	4.7	1.0–19.0	9.3	0.0	2.3
	29	Johnston	41	85.4	4.9	1.0–19.8	9.8	0.0	2.4
	38	Craven	54	94.4	0.0	–	5.6	0.0	3.7
	39	Craven	51	74.5	19.6	9.7–35.7	5.9	0.0	0.0
	40	Craven	46	52.2	47.8	32.4–63.7	0.0	0.0	4.4
	41	Jones	47	72.3	27.7	15.8–43.9	0.0	0.0	0.0
	42	Jones	43	95.4	4.7	1.1–17.7	0.0	0.0	11.6
	43	Jones	42	7.1	92.9	78.7–97.9	0.0	0.0	0.0
Coastal Plain total			1081	81.7	15.4	12.8–18.3	2.6	0.4	3.1
Tidewater									
	2	Washington	16	100.0	0.0	–	0.0	0.0	0.0
	3	Beaufort	40	97.5	0.0	–	2.5	0.0	0.0
	8	Washington	48	97.9	0.0	–	2.1	0.0	0.0
	9	Washington	52	78.9	17.3	8.2–32.9	3.9	0.0	0.0
	10	Beaufort	31	100.0	0.0	–	0.0	0.0	0.0
	49	Chowan	50	96.0	4.0	0.9–15.5	0.0	0.0	0.0
	50	Chowan	56	96.4	1.8	0.2–12.3	1.8	0.0	3.6
	51	Chowan	46	84.8	15.2	6.9–30.4	0.0	0.0	2.2
	52	Pasquotank	47	80.9	19.2	9.5–34.7	0.0	0.0	2.1
	53	Pasquotank	35	74.3	25.7	13.0–44.5	0.0	0.0	2.9
	54	Perquimans	35	94.3	5.7	1.4–21.2	0.0	0.0	0.0
	55	Perquimans	40	92.5	7.5	2.3–22.2	0.0	0.0	0.0
	56	Perquimans	50	94.0	6.0	1.8–18.2	0.0	0.0	4.0
	57	Perquimans	55	89.1	7.3	2.4–20.2	3.6	0.0	0.0
Tidewater total			601	90.7	8.2	5.9–11.2	1.2	0.0	1.2
Total			2197	86.2	11.3		2.2	0.3	2.2

[a] FSAMSC = *Fusarium sambucinum* species complex, FTSC = *Fusarium tricinctum* species complex, FFSC = *Fusarium fujikuroi* species complex, FIESC = *Fusarium incarnatum-equiseti* species complex.
[b] 95% confidence interval (CI) calculated using Goodman's intervals; see text for details.

[c] Nivalenol chemotype of *F. graminearum* isolates, which are also included under FSAMSC. The vast majority of *F. graminearum* isolates had the 15-ADON chemotype; only 8 isolates (0.4%) were of 3-ADON chemotype across entire sample.

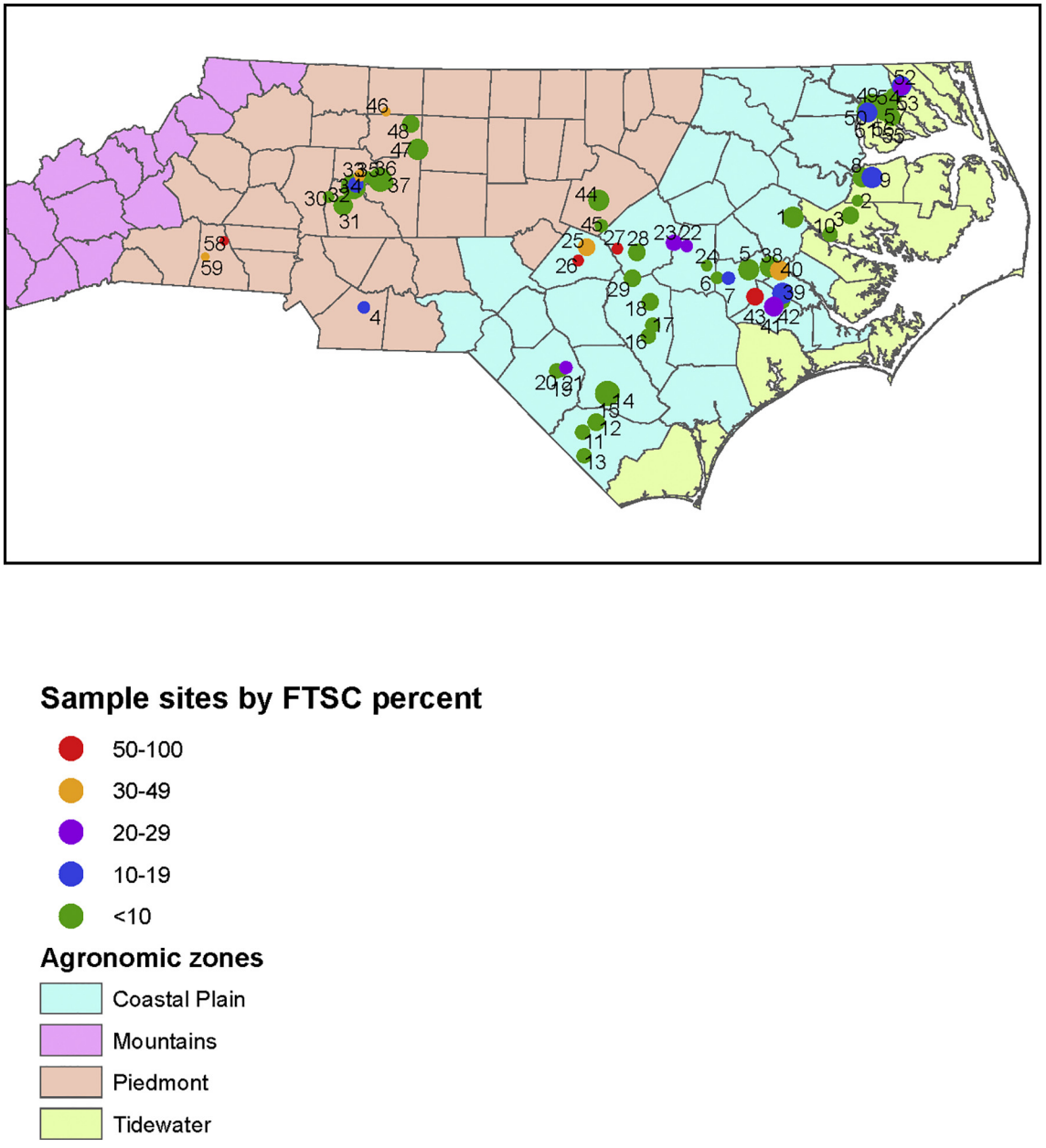

Fig. 1. Locations of 59 North Carolina fields in three agronomic zones where FHB-symptomatic wheat spikes were sampled in May 2014. Field numbers correspond to those in Table 1. Marker diameters are proportional to sample sizes, and marker colors indicate percent of derived isolates belonging to the *Fusarium tricinctum* species complex, which produce the "emerging mycotoxins" moniliformin and enniatins, and do not produce trichothecenes.

2. Materials and methods

2.1. Sample collection and isolate derivation

Wheat spikes symptomatic for FHB were sampled from 59 commercial fields in 24 North Carolina counties in the 2013–14 growing season (Table 1). Of the fields, 14 were located in the Tidewater zone, 29 in the Coastal Plain, and 16 in the Piedmont (Fig. 1). The zones (NCpedia, 2019) are commonly accepted in the mid-Atlantic and Southeast U.S. as distinct agronomic regions, and for the present purpose, counties that were mainly in one zone were assigned entirely to that zone (Fig. 1). The goal was to sample three fields in each of the major wheat-producing counties of the state, but in some of those counties, only one or two symptomatic fields could be located. North Carolina experienced low to moderate FHB incidence in 2014, with occasional severely affected fields. FHB was relatively more frequent in the Tidewater and Coastal Plain, and less frequent in the Piedmont that season due to rainfall patterns in April and May (C. Cowger, personal observations).

During drives along roads through agricultural areas, fields were chosen for sampling when the winter wheat crops exhibited symptoms of FHB. All the crops were likely soft wheat, but the varieties of wheat and the previous crops were unknown. Collection occurred during the period of 13 and 28 May 2014, corresponding to the dates when symptoms of spike bleaching were clearly visible and samples could be gathered across the state. Symptomatic spikes were collected from each

field while walking a random path through as large an expanse as possible. While a sample of at least 30 symptomatic spikes per field was sought, there were cases in which only smaller numbers could be collected due to infrequency of symptoms.

To isolate *Fusarium* strains from spikes, one infected spikelet per spike was surface-disinfected in 2% sodium hypochlorite (bleach) for 1 min, rinsed in sterile water, and plated on a modified Nash-Snyder *Fusarium*-selective medium (Schmale III et al., 2006). After 4 to 5 days, transfers from the resulting culture were made to a quarter-strength potato dextrose agar (PDA) plate. One single-spored isolate per wheat spike was chosen for use in the subsequent analysis. That single genetic individual was grown in potato dextrose broth for 2–4 days on a rotating shaker at 150 rpm. The mycelium was harvested and lyophilized.

DNA was extracted from each lyophilized sample by means of a simple bead-beating procedure. Approximately 15 mg of lyophilized mycelium was ground in a 1.5-ml microcentrifuge tube by vortexing for 30 s with 10 nickel-plated lead shot beads. DNA was extracted from the ground tissue using an EZNA Plant DNA Mini kit (Omega Biotek, D2485). DNA concentration was determined using a Qubit dsDNA HS assay kit (ThermoFisher, Q32854).

2.2. Genotyping

Isolate characterization began with PCR assays to amplify two genes, *TRI3* and *TRI12*, as described in Ward et al. (2002) and Starkey et al. (2007). *TRI3* encodes a 15-*O*-acetyltransferase (McCormick et al., 1996) and *TRI12* encodes a trichothecene efflux pump (Alexander et al., 1999); the assays were designed to produce chemotype-specific amplicons from the FGSC and closely related species within FSAMSC lineage 1 (FSAMSC-1, (Kelly et al., 2016). However, a significant number of isolates processed with the *TRI3* and *TRI12* primers did not produce bands of the expected sizes, or produced multiple bands. Because this suggested that isolates from outside FSAMSC lineage 1 might be present, a combination of multilocus genotyping (MLGT) and DNA sequencing were employed for species identification of all isolates.

Species identity and trichothecene type were determined via MLGT as described previously (Sarver et al., 2011; Ward et al., 2008) using a 50-probe assay that enabled simultaneous identification of 21 FSAMSC-1 species as well as the 15-ADON, 3-ADON, NIV, and NX trichothecene types (Garmendia et al., 2018). Isolates that could not be identified by MLGT were analyzed using partial sequences of the translation elongation factor 1α (*TEF1*) as described previously (Cerón-Bustamante et al., 2018). Sequence similarity searches were performed using the FUSARIUM MLST database (http://www.westerdijkinstitute.nl/fusarium/), with species identifications based on > 99% similarity between query and reference sequences. Isolates that produced lower similarity scores in relation to reference sequences were identified to the level of species complex based on the best sequence matches in FUSARIUM MLST. FUSARIUM MLST results were cross-checked in relation to the non-redundant nucleotide collection database at NCBI.

2.3. Mycotoxin analyses

To assess mycotoxin content of the samples, the spikes that remained after an isolate had been derived from one spikelet per spike were utilized. Approximately 30 spikes per field were bulked together and ground to flour with a Laboratory Mill 3100 (Perten Instruments, Hägersten, Sweden). One gram of each ground wheat sample was extracted with 10 ml of an acetonitrile/water mixture (86:14) for 15 min in a 50 ml Falcon tube with shaking on a horizontal vortex genie. The mixture was centrifuged for 5 min to pellet the plant material.

To measure trichothecene concentrations, gas chromatography–mass spectrometry (GC-MS) was used. Five ml of the acetonitrile/water extract was purified with a Romer MycoSep cleanup column. Two ml of the purified extract was dried in a 1-dram vial with heat under a stream of nitrogen. 100 µl of freshly prepared silylating reagent (1-(trimethylsilyl)imidazole/chlorotrimethylsilane, 100:1) was added to the vial and the tube was quickly vortexed to coat the walls and then incubated for 30 min. 900 µl of isooctane were added to the vial and briefly vortexed to mix. One ml of water was then added to the vial to quench the reaction, and the vial was gently shaken until the top layer was clear. The top organic layer was then transferred to a GC vial for GC-MS analysis.

GC-MS analyses were performed with an Agilent 7890 chromatograph (Wilmington, Delaware) fitted with a HP-5MS column (Wilmington, Delaware) and products detected with an Agilent 5977 mass spectrometer with an electron impact source operating in selected ion monitoring (SIM) mode. Samples were introduced with splitless injection at 150 °C, the temperature was held for 1 min and then the column was heated at 30 °C/min to 280 °C and then held for 1 min. Under these conditions, 3,7,15-tri-trimethylsilyl DON is detected at 6.2 min. For quantitation of DON, samples were run in selected ion monitoring (SIM) mode using ions 512, 422, and 392, 295, 259, and 235. DON was quantified using a standard curve of 3,7,15-tri(tri-trimethylsilyl) DON derivatives prepared in the same way.

To assess non-trichothecene mycotoxins not detected by GC-MS, the remaining 5 ml of acetonitrile/water extract was analyzed by high-performance liquid chromatography-mass spectrometry (LC-MS). Here the goal was particularly the detection and measurement of toxins produced by the FTSC: beauvericin, enniatins, moniliformin and 2-AOD-ol (2-amino-14,16-dimethyloctadecan-3-ol), a sphingolipid-like metabolite. The analysis was performed with a Dionex Model U3000 liquid chromatography system (Thermo Scientific, Waltham, Massachusetts), and a QExactive mass spectrometer (Thermo Scientific). The LC-MS analysis was conducted in full scan positive electrospray ionization (ESI) mode to observe a wide range of metabolites and in negative ESI mode specifically for the detection of the mycotoxin moniliformin. Positive mode mass spectrometry was done with a gradient of 20–95% aqueous methanol over 5 min on a Phenomenex Kinetex XB-C18 column (2.1 mm × 50 mm) at a flow rate of 0.6 ml/min. For positive mode operation, LC mobile phases were modified with 0.3% acetic acid to aid chromatographic separation and MS detection. Negative mode mass spectrometry was done with a gradient of 5–95% aqueous methanol over 5 min on a Waters XBridge C18 column (4.8 mm × 150 mm) at a flow rate of 0.8 ml/min. For negative mode operation, LC mobile phases were modified with 0.1% formic acid to aid chromatographic separation and MS detection.

The limits of quantitation for the secondary metabolites evaluated were: DON, 0.2 µg g^{-1}; 2-AOD-ol, 10 µg g^{-1}; beauvericin, 0.4 µg g^{-1}; enniatins, 0.15 µg g^{-1}; and moniliformin, 10 ng g^{-1}.

2.4. Statistical analysis

To determine whether FTSC percentages in fields or zones differed from each other, 95% confidence intervals were calculated around the FTSC percentages using the Goodman method for multinomial proportions (Goodman, 1965; May and Johnson, 1997). This method performs well when cell frequencies are not < 5 and categories are few. It produces a nonsymmetrical interval with a restricted range within the interval [0–1] or [0–100%]. For comparing the proportions of a given species complex in different zones, the presence of non-overlapping confidence intervals for two zones was used as statistical evidence ($\alpha = 0.05$) of a nonzero difference between those zones. SAS software version 9.4 was used to run the analyses, with the code based on the SAS macro program (blog 'DOloop', https://blogs.sas.com/content/iml/2017/02/15/confidence-intervals-multinomial-proportions.html).

3. Results

From 59 fields in the three agronomic zones, a total of 2197 isolates were identified to species (Table 1). The number of isolates identified per field ranged from 9 to 66.

3.1. Species identities

Across the entire sample of 2197 isolates, a large majority of isolates (86.2%) were members of the trichothecene-producing FSAMSC (Table 1). Out of the total, 1821 or 83% possessed the *F. graminearum* 15-ADON genotype. Only 48 NIV-genotype isolates were identified among the *F. graminearum* isolates, and they were widely scattered, with one or more NIV-type isolates found in 26 of the 59 fields. The per-field percentage of NIV isolates ranged from 0 to 12%, with 12% found in fields 14 (Bladen County) and 42 (Jones County) in the Coastal Plain. Agronomic zones did not differ for NIV percentage, with the highest rate being 3% of isolates in the Coastal Plain, which was not significantly different from 1.6% in the Piedmont and 1.2% in the Tidewater ($P > .05$). Just nine *F. graminearum* isolates of the 3-ADON genotype (0.4% of the total sample) were found. Besides *F. graminearum*, a small handful of isolates were identified as other species belonging to the FSAMSC: five *F. poae*, three *F. armeniacum*, and two *F. sporotrichioides* isolates.

Again across the entire sample, 249 isolates (11.3%) were classified in the FTSC (Table 1). However, there was a wide range in FTSC frequencies among fields. The high of 92.9% was found in field 43 in Jones County, located in the Coastal Plain zone; this percentage was significantly higher than those in all but two other fields (Fields 26 and 27, also in the Coastal Plain, which had 58.8% and 63.2%, respectively). Altogether, there were four fields in the 50–100% FTSC range, five in the 30–49% range, five in the 20–29% range, and seven in the 10–19% range (Table 1, Fig. 1). The remaining 38 fields had FTSC percentages below 10%.

Many of the fields with relatively high FTSC percentages were concentrated in a belt stretching across six counties in the central Coastal Plain, between latitudes 35.0 and 35.5, where there were nine fields with FTSC percentages of ≥20% (Fig. 2). There were also four fields in the Piedmont with FTSC percentages of ≥20%, and one in the Tidewater zone. When comparing zones, however, the FTSC percentage was significantly higher in the Coastal Plain at 15.4% than in the Piedmont (6.6%) or Tidewater (8.2%) (Table 1).

Of the 249 FTSC isolates, 122 were *F. acuminatum*, 59 were *F. reticulatum*, 5 were *F. avenaceum*, 51 belonged to an unnamed species in FUSARIUM MLST (FTSC11) that is closely related to *F. avenaceum* (Moreira et al., 2020; O'Donnell et al., 2018), and 12 were classified simply as FTSC because they did not share at least 99% similarity with reference sequences in FUSARIUM MLST.

Out of the total of 2197 isolates, 48 belonged to the *Fusarium fujikuroi* species complex, or FFSC (Aoki et al., 2014; O'Donnell et al., 2015); of them, 45 were *F. proliferatum*, one was *F. concentricum*, and two others were classified simply as FFSC. Per-field percentages were usually < 10% FFSC, although one field (Field 30) had 28% *F. proliferatum* (Table 1). Six isolates were classified as members of the *Fusarium incarnatum-equisetum* species complex, or FIESC (O'Donnell et al., 2015).

3.2. Mycotoxin content

The whole-spike samples had DON concentrations ranging from 0.77 to 152.78 μg g^{-1} (Table 2), as measured by GC-MS. The samples had moniliformin (MON) concentrations ranging from 0.0 to 38.73 μg g^{-1}, with most values being < 1.0 μg g^{-1}, as measured by LC-MS. While no quantifiable levels of enniatins (ENNs) were detected in most samples, a few samples did contain levels of 0.19 to 0.31 μg g^{-1} of ENN B, ENN B1, or both. No other FTSC toxins (beauvericin, chlamydosporol, ENN A, ENN A1, or 2-AOD-ol) were detected, and no butenolide (an FGSC toxin) was detected.

The samples with the highest MON levels, i.e., concentrations above 3.0 μg g^{-1}, came from fields in which at least 50% of isolates were identified as members of the FTSC (Fig. 3). Although the set of fields with ≥50% FTSC strains was small, within it the relationship of FTSC percentage to MON was roughly linear. However, the sample from Field 27 with 63% FTSC isolates had a low MON concentration (0.56 μg g^{-1}), so a high FTSC percentage was apparently a necessary but not sufficient condition for a relatively high MON level in these samples.

The highest DON levels, i.e., concentrations of 40 μg g^{-1} and above, were found where FSAMSC percentage was 95–100%, although there were also fields with FSAMSC percentages in that range that had lower DON levels (Tables 1 and 2). The three fields with the highest MON concentrations all had relatively low DON levels (4.2–6.3 μg g^{-1}). This was reasonable, given that those fields also had relatively high FTSC

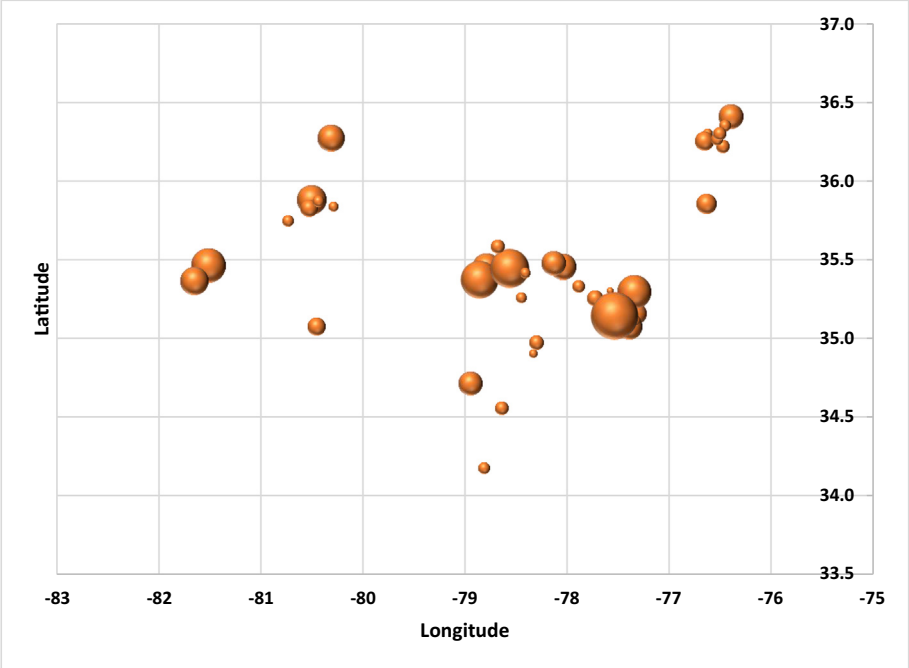

Fig. 2. Latitude and longitude of 59 North Carolina wheat fields sampled for FHB-symptomatic spikes in 2014, with marker diameter corresponding to the proportion of spikes infected with isolates in the *Fusarium tricinctum* species complex.

Table 2
Concentrations of deoxynivalenol (DON), moniliformin (MON), and total type B enniatins (ENNs) in FHB-symptomatic spike samples from 59 North Carolina wheat fields in 2014.

Field	County	DON (ug g^{-1})	MON (ug g^{-1})	ENNs (ug g^{-1})[a]	Field	County	DON (ug/g)	MON (ug/g)	ENNs (ug g^{-1})[a]
1	Pitt	0.77	0.24	–	31	Rowan	25.41	0.17	–
2	Washington	18.10	0.15	–	32	Rowan	9.85	0.24	–
3	Beaufort	8.17	0.14	0.25	33	Davie	4.88	0.29	–
4	Union	20.91	0.24	–	34	Davie	4.58	0.34	–
5	Lenoir	10.14	0.16	0.25	35	Davie	4.97	0.33	–
6	Lenoir	19.33	0.21	–	36	Davidson	58.52	0.95	–
7	Lenoir	18.12	0.19	0.55	37	Davidson	7.18	0.43	–
8	Washington	55.76	0.00	–	38	Craven	4.73	0.19	–
9	Washington	30.82	0.20	–	39	Craven	20.32	1.35	0.24
10	Beaufort	64.90	0.00	0.19	40	Craven	4.62	0.46	–
11	Columbus	3.91	0.04	–	41	Jones	13.37	0.21	–
12	Columbus	10.07	0.08	0.46	42	Jones	47.07	0.41	–
13	Columbus	12.80	0.69	–	43	Jones	4.61	38.73	0.43
14	Bladen	8.97	0.57	–	44	Wake	27.93	0.40	–
15	Bladen	10.25	0.33	–	45	Wake	6.38	0.49	–
16	Sampson	13.58	0.11	0.22	46	Stokes	8.51	0.09	–
17	Sampson	13.92	1.63	–	47	Forsyth	39.51	0.16	–
18	Sampson	18.03	0.17	–	48	Forsyth	152.78	0.20	–
19	Robeson	8.78	0.14	–	49	Chowan	17.77	0.10	–
20	Robeson	6.87	0.21	–	50	Chowan	8.07	0.09	–
21	Robeson	11.57	0.18	–	51	Chowan	20.69	1.38	0.19
22	Wayne	4.82	0.17	–	52	Pasquotank	8.26	0.88	0.19
23	Wayne	9.31	0.30	–	53	Pasquotank	10.02	0.83	–
24	Wayne	5.61	0.07	0.22	54	Pasquotank	2.61	0.14	–
25	Harnett	32.58	0.18	–	55	Perquimans	5.10	0.15	–
26	Harnett	4.16	21.55	0.31	56	Perquimans	30.00	0.48	–
27	Johnston	20.72	0.56	–	57	Perquimans	28.62	0.10	–
28	Johnston	12.97	0.23	–	58	Cleveland	6.33	3.69	–
29	Johnston	21.99	0.15	0.29	59	Cleveland	2.33	0.06	–
30	Rowan	2.09	0.18	–					

[a] Sum of type B and type B1 enniatins; "–" indicates below limits of quantification.

and low FSAMSC percentages (Table 1).

3.3. Previous crop

Although the previous crop was not identified at the time of sampling the FHB-symptomatic wheat spikes, it was determined retrospectively for five fields with 0% FTSC and five fields with FTSC of ≥30% by contacting farm managers. The previous crops in the 0% FTSC fields were soybeans (two fields) and cotton, tobacco, and maize (one field each). The previous crops in the high-FTSC fields were tobacco (two fields), soybeans (two fields), and either fescue or sudex, a sorghum-sudangrass hybrid (one field).

4. Discussion

This study provides a detailed look at the distribution of FHB-causing strains in commercial winter wheat crops at the level of a single U.S. state. To our knowledge, this is the largest and most intensive survey of *Fusarium* strains within an FHB-prone wheat area of its size. The state utilizes crop rotations common to many eastern U.S. states, in

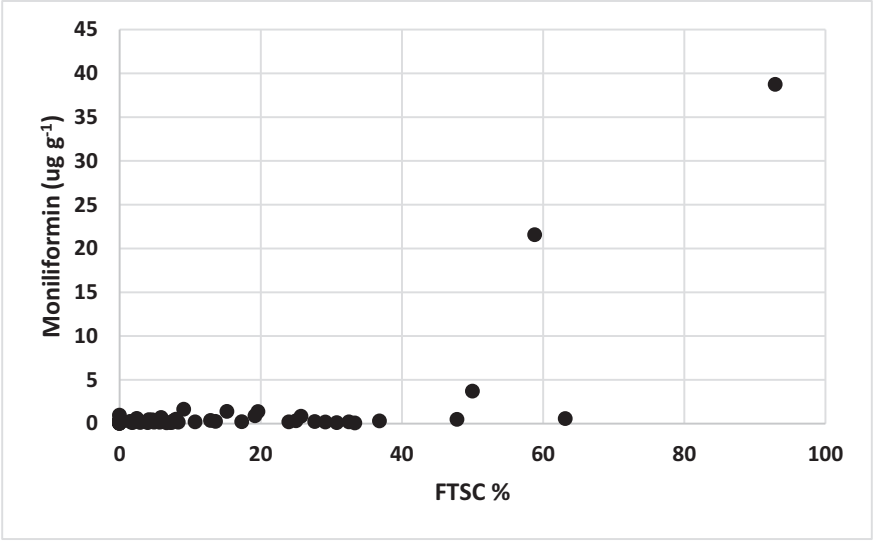

Fig. 3. Concentrations of moniliformin in samples from 59 North Carolina wheat fields, in relation to percent of sampled spikes yielding a strain in the *Fusarium tricinctum* species complex.

particular that of corn, soybeans and wheat. Overall, the present results confirmed that *F. graminearum* was the dominant FHB-causing pathogen in wheat at the scale of state. Unexpectedly, however, in some individual wheat fields within North Carolina there were sizable minority populations of FTSC species, and in three fields those species constituted the majority of the sample.

The results suggest it may be useful to begin taking these producers of "emerging mycotoxins" into account in managing FHB in the U.S. It seems unlikely the patchy dominance of FTSC FHB is confined to the year 2014 or to North Carolina, as the state's agricultural production system is reasonably stable over time, and its climate and crop rotation patterns are similar to those in surrounding areas. In 2014, North Carolina producers harvested a little over 311,600 ha of wheat, 315,600 ha of maize, and 700,000 ha of soybeans, while in the four adjacent states, a total of between 489,500 and 1.2 million hectares of each of those same crops was harvested that year (NASS, 2019). The present study demonstrates that in this production environment, FHB-causing species can vary by field even when fields are near each other, and sampling must be on a fairly fine scale in order to detect wheat fields where minority species are at higher frequencies.

The fact that the importance of FTSC strains in some small-grain fields was not noticed before now is likely due to limited sampling, and also to a focus on characterization of genetic and mycotoxin diversity among isolates of the dominant FHB pathogen, *F. graminearum*, and closely related species. It should be noted that the *TRI3* and *TRI12* PCR assays, commonly used for chemotype prediction, are only appropriate for characterization of *F. graminearum* and other members of FSAMSC-1. While those primers reliably distinguish among strains with 15-ADON, 3-ADON, or NIV chemotypes, they do not produce accurate results for species outside FSAMSC-1. For example, spurious amplification of non-target DNA from many of the non-FGSC isolates in this study produced amplicons that were similar in size to the expected amplicon from 3-ADON strains, which could lead to false conclusions if interpreted in the absence of accurate species identifications. In addition, these primers were designed prior to the discovery of the NX toxin type among *F. graminearum* and can result in the misidentification of NX isolates as having the 3-ADON chemotype. MLGT provides for simultaneous identification of species and trichothecene type based on direct interrogation of species or chemotype-specific SNPs genotypes, and includes clade-specific probes for FSAMSC-1 and the FGSC, eliminating the potential issues of misinterpretation based on spurious amplification of non-target DNA. The latest version of MLGT (Garmendia et al., 2018) also includes probes providing for reliable identification of NX isolates.

As expected, the percentages of NIV-producing *F. graminearum* strains and those producing 3-ADON were very small. No field had a high concentration of NIV producers, which indicates there is no reason to routinely monitor NIV levels in winter wheat in this U.S. region, in line with current practice. The scattered small percentages are consistent with the results from the previous, much smaller survey (Schmale et al., 2011).

What explains such wide variation in FTSC frequency in wheat fields that lie in close proximity? One possible factor is localized weather conditions, which may favor some FHB-causing strains over others. For example, the timing of FHB-conducive conditions in relation to wheat anthesis in certain fields may have favored *F. acuminatum* or other members of the FTSC in relationship to *F. graminearum*. Comparing the performance of *F. graminearum, F. acuminatum* and *F. avenaceum* when individually infecting wheat spikes at different timings during anthesis, Beccari et al. (2019) found that *F. graminearum* caused greater and more rapid symptom development than the weaker pathogens after all infection timings. But the *F. graminearum*:FTSC ratios of biomass and secondary metabolites were higher from inoculations at 3 or 6 days after early anthesis (daa) than at 0 or 9 daa. Thus, perhaps rain that results in spore release either at the start of wheat anthesis or late in anthesis gives a relative advantage to FTSC strains in competition with *F. graminearum*. It should be noted that the study by Beccari et al. (2019) did not test co-occurrence within spikes, and was in a controlled environment, so these dynamics remain to be tested in field situations.

Another weather factor that may affect competition among fusaria is temperature. In the UK and Europe, where multiple FHB-causing pathogens including *F. graminearum*, *F. poae*, and *F. avenaceum* are often present in wheat fields, *F. graminearum* was associated with warmer and *F. avenaceum* with cooler conditions (Xu et al., 2008). It is possible that in the present study, fields with relatively high percentages of FTSC strains experienced cooler micro-climatic conditions than other fields during key stages of infection and disease development.

Previous crop is another possible explanation for differences among fields. There is some evidence that previous crop can influence the relative frequencies of FGSC chemotypes (Pasquali and Migheli, 2014). As is discussed below, moniliformin contamination is thought to be more prevalent where maize is extensively cultivated (Peltonen et al., 2010). In the present study, no pattern among previous crops was apparent in the subset of sampled fields that generated either low or high FTSC percentages. Perhaps a larger dataset on previous crop would have detected tendencies that were not evident from this subset, but due to the difficulty of locating farm managers with relevant information several years later, this would have been extremely difficult to recreate in hindsight.

FTSC percentages were on average about twice as high in the Coastal Plain as in the Piedmont or Tidewater zones, and it is unclear what factors account for this. The Coastal Plain generally possesses a sandy loam soil, in contrast to the clay and mineral organic soils of the other two regions, respectively. However, no data are available to assess if FTSC species are favored by lighter, sandier soils.

While it has not been identified as an important agent of FHB in the USA, *F. acuminatum* has been implicated in soilborne diseases of field crops. For example, *F. acuminatum* and *Cochliobolus sativus* were considered primary agents in the root rot complex of dryland winter wheat in Colorado and Wyoming (Hill and Fernandez, 1983). *F. acuminatum* was also among the top three *Fusarium* species isolated from soybean roots in Iowa (Diaz-Arias et al., 2013) and southern Alberta, Canada (Zhou et al., 2018). Possibly the large-scale soybean production in the eastern and central U.S. provides a source of *F. acuminatum* inoculum for FHB. Then, in a given field, competitive dynamics among species complexes may be influenced by environmental factors such as moisture, temperature, and the frequencies of other micro-organisms.

What are the implications of the present findings for mycotoxin management? Generally, the main toxin of concern in U.S. small grains is DON, and the current results were consistent with that. However, the study also demonstrated the potential for occurrence in mid-Atlantic wheat crops of what are known as "emerging mycotoxins" produced by FTSC strains *F. avenaceum* and *F. acuminatum*, in particular moniliformin and the depsipeptides beauvericin and enniatins. In the U.S., there is no federal advisory level for these mycotoxins, and their impact on consumer health remains unclear.

Moniliformin (MON), which was detected and quantified in this study, is a mycotoxin found in maize, wheat, barley and oats; it often co-occurs with other *Fusarium* toxins (Knutsen et al., 2018). It has been detected in Canadian samples of wheat, oats and rye, with the highest concentrations generally in durum wheat (Clear et al., 2005; Gräfenhan et al., 2013; Tittlemier et al., 2013), and also in small-grain cereals in Austria, Finland, Norway and Poland (e.g., (Filek and Lindner, 1996; Jestoi et al., 2004; Sharman et al., 1991; Uhlig et al., 2004). While the cytotoxicity of MON is relatively low, its acute toxicity is comparable to that of the most toxic *Fusarium* trichothecenes such as T-2 toxin, and particularly affects the heart in various animals (Hallas-Møller et al., 2016; Peltonen et al., 2010). Adverse effects from chronic MON consumption include mortality and reduced body weight gain in swine and poultry, reduced weight gain and body weight in mink and farmed fish, and myocardial lesions and reduced egg production in poultry (Knutsen

et al., 2018; Manning and Abbas, 2012; Peltonen et al., 2010). Due to a lack of data, the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain could not establish a tolerable daily MON intake for animals or humans (Knutsen et al., 2018). Overall, the EFSA Panel concluded that current feeding practices create a low or even negligible risk to farm and companion animals, and there is likely a low risk to human health from chronic consumption of levels of MON currently found in grain products. However, the EFSA Panel also noted the very limited quantity of available dose-response data, especially on cardiotoxicity, and recommended a 90-day toxicity study be conducted with rats.

When MON is detected in small-grain cereals, enniatins (ENNs) and beauvericin (BEA) are often also detected. *F. acuminatum* and *F. avenaceum* strains have various patterns of mycotoxin production, depending on geographic region, climate, substrate of origin, and test substrate (Jestoi et al., 2004; Logrieco et al., 1992). They may produce MON, ENNs, BEA, and other mycotoxins in different combinations. MON and ENNs have co-occurred in other surveys of small-grain samples where FTSC strains were found, e.g., in Finland (Jestoi et al., 2004; Tittlemier et al., 2013; Yli-Mattila et al., 2006). In the present study, low concentrations of type B ENNs were detected in some samples, while no quantifiable levels of type A ENNs or BEA were found. The possible co-occurrence of ENNs with MON in U.S. wheat fields merits further investigation.

The present results demonstrate the possibility of mycotoxin mixtures in US wheat fields. There is growing interest in the effects of mycotoxin mixtures, as toxic effects can occur when multiple toxins are present even though no single mycotoxin is above its allowable ceiling (Silva et al., 2002). MON may be of concern due to interactive effects when it is combined with other *Fusarium* mycotoxins such as fumonisin B_1 or DON, even when the levels of each individual toxin do not exceed known thresholds (Fremy et al., 2019). Moreover, MON can be produced by nearly 40 *Fusarium* species (Peltonen et al., 2010) as well as *Penicillium melanoconidium*, a post-harvest contaminant of stored cereals (Hallas-Møller et al., 2016). Also, MON is commonly found in other crops grown in rotation with wheat, especially in maize (Gutema et al., 2000; Peltonen et al., 2010), where it is produced by *F. subglutinans*, particularly in warmer areas, or *F. proliferatum* (Desjardins et al., 2006; Peltonen et al., 2010). Thus, MON contamination in feed made from multiple cereal grains (e.g., maize and wheat) could exceed a tolerable ceiling even if the MON level in each component feedstock was lower.

There are few published observations of FTSC mycotoxin concentrations in commercial North American grain crops. In one report, Canadian durum wheat samples infected with both *F. graminearum* and *F. avenaceum* contained a mean of 3.8 μg g^{-1} of depsipeptides, concentrations of which were up to 10 times higher than those of MON or DON (Tittlemier et al., 2013). Durum grain samples containing higher concentrations of both MON and depsipeptides were also graded correspondingly lower, indicating that *Fusarium*-damaged kernels (FDK), which are known to correlate with DON, also correlated with the *F. avenaceum*-generated mycotoxins. Clearly, more sampling would need to take place in North Carolina wheat fields to clarify what if any levels of contamination with MON or depsipeptides might be occurring.

A limitation of this study was the selection of a single isolate per spike, which did not allow the detection of multiple species or strains that may have co-occurred within a spike. Given the large scale of the effort and resource limitations, it was judged of greater interest to sample more extensively within fields rather than intensively within spikes. In future, it would be helpful to determine the degree to which individual symptomatic wheat spikes are colonized by FHB-causing strains of multiple species. It is noteworthy that high MON concentrations were only observed in symptomatic spike samples with ≥50% FTSC percentages and relatively low DON levels. This suggests, although does not demonstrate, that there may be limited or highly unequal *F. graminearum*-FTSC co-occurrence in individual spikes. Little is known about co-infection of individual wheat spikes by multiple *Fusarium* species.

A study of two wheat fields in the Netherlands found that spike residues and grain in each field were both colonized at harvest by multiple fusaria (*F. avenaceum*, *F. culmorum*, *F. graminearum*, and *F. poae*) as well as *Microdochium nivale* (Köhl et al., 2007), although spikes were pooled and not assayed individually. In the Dutch experiment, all the species were also found in leaves and stems, and their relative proportions varied across the seasons of a full year. This reminds us that relative inoculum availability of different fusaria at wheat flowering reflects the outcome of saprophytic competition on wheat, maize, and other stubble.

What implications do these results have for breeding and deployment of resistant cultivars? It has been suggested, although not demonstrated, that resistance effective against one FHB-causing pathogen may be effective against others, whether within the FGSC (Pasquali and Migheli, 2014) or within the FSAMSC when cultivar responses to *F. culmorum* and *F. graminearum* were compared (van Eeuwijk et al., 1995). Moving beyond the trichothecene producers, there is little information on whether resistance to FGSC strains is equally effective against strains from other species complexes, such as those of the FTSC. Thus, it remains to be determined whether a breeding nursery that utilizes natural inoculum in a high-FTSC location would obtain the same resistance rankings of genotypes as a nursery in a high-FGSC location.

At a practical level, *F. graminearum* and trichothecene mycotoxins remain the dominant concern in managing FHB in North Carolina. The evidence indicates that NIV producing strains are highly infrequent, and monitoring NIV in grain crops is not warranted. However, FTSC species can be significant causal agents of FHB epidemics in this environment. Coastal Plain small grain fields appear to have a somewhat greater likelihood than those in other zones of generating higher percentages of FTSC-infected wheat spikes. The patchy distribution of FTSC species suggests a need to better understand the factors that sometimes allow them to outcompete trichothecene producers, as well as the distribution and importance of their mycotoxins in cereal crops. A clearer picture of what drives the FHB-causing species balance toward the FTSC may be useful in improving host resistance and/or management practices so as to minimize both sets of pathogens.

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