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A high proportion of NX-2 genotype strains are found among *Fusarium graminearum* isolates from northeastern New York State

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Abstract *Fusarium graminearum*, a fungal pathogen of wheat, barley, and corn, produces a variety of trichothecene mycotoxins that are important as virulence factors and as seed contaminants reducing grain quality. A previous survey of the pathogen in New York State identified variation in genes indicative of trichothecene diversity. Recently *F. graminearum* strains that produce a newly characterized trichothecene mycotoxin called NX-2 have been identified in North America. Using a large collection of *F. graminearum* strains from Willsboro NY, we found that the frequency of NX-2 genotype strains was 7–14 times higher than at other locations where it was reported previously. NX-2 genotypes were not only found in wheat heads but also found in high frequency from air samples and on maize ears and stubble. Because NX-2 genotypes may represent as much as 20% of the total *F. graminearum* population, this regional fungal population provides an opportunity to assess the effects of the novel NX-2 trichothecene on fungal virulence, toxin loading, and patterns of host specificity that could inform future disease management and plant breeding.

Keywords Fusarium head blight · NX-2 · Mycotoxin · *Fusarium graminearum*

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Introduction

Fusarium graminearum sensu stricto (O'Donnell et al. 2004) is an important cause of stalk rot of maize as well as Fusarium head blight of wheat and barley in North America and worldwide (Starkey et al. 2007; Kelly et al. 2015, 2016). In addition to causing disease, the fungus also contaminates grain with trichothecene mycotoxins such as deoxynivalenol (DON), also known as vomitoxin. DON is a demonstrated virulence factor for Fusarium head blight on wheat (Proctor et al. 1995;

Jansen et al. 2005) and can cause acute toxicosis when ingested in sufficient amount, characterized by gastrointestinal distress, hemorrhage and, ultimately, death (Pestka 2010). Most developed nations monitor trichothecene levels in grain with the goal of limiting human dietary intake of DON.

While DON is the major trichothecene mycotoxin that accumulates in wheat and barley grain, considerable naturally occurring variation in trichothecene structures exist. Strains of *F. graminearum* generally produce one of four different trichothecene profiles, or chemotypes and these chemotypes are determined by allelic differences in the fungal genes encoding enzymes in the trichothecene pathway (Rep and Kistler 2010). These chemotypes include strains that produce predominantly 7-hydroxy, 8-keto trichothecenes in culture; either 3-acetyl deoxynivalenol (3ADON), 15-acetyl deoxynivalenol (15ADON) or nivalenol (NIV) (Miller et al. 1991; Ward et al. 2002). The trichothecenes 3ADON and 15ADON produced by the fungus can be further metabolized by host plants to DON (Schmeitzl et al. 2015a, b). More recently *F. graminearum* strains producing a novel 7-hydroxy, 8-deoxy trichothecene structure have been identified. This new trichothecene has been called NX-2, and the structure of the molecule is identical to that of 3ADON except for the absence of a keto group at the 8 position carbon (Varga et al. 2015). NX-2 strains never make DON but rather accumulate the compound equivalent to DON *in planta* (called NX-3) that lacks the keto group at the 8 position carbon (Varga et al. 2015). NX-2 strains have only been reported in Canada and the northern United States and have thus far only been found at low frequencies (<3%) (Liang et al. 2014; Kelly et al. 2015, 2016).

Chemotype differences for the 7-hydroxy, 8-keto trichothecenes are determined by polymorphic genes within the major trichothecene biosynthetic gene cluster on chromosome 2 (Brown et al. 2002; Lee et al. 2002; Rep and Kistler 2010); this knowledge was used to design PCR based assays for identifying 3ADON, 15ADON and NIV chemotype strains (Ward et al. 2002; Quarta et al. 2006). Using such assays, numerous surveys for chemotype have been conducted worldwide (van der Lee et al. 2015) including in New York State (Kuhnem et al. 2015b). However, these PCR based assays do not distinguish 7-hydroxy, 8-keto trichothecene chemotypes from the NX-2 chemotype because the allele encoding hydroxylation at the 7 position carbon alone, instead of both the 7 and 8 position carbon, is encoded at the Tri1 locus on chromosome 1 (Kelly et al. 2016; Varga et al. 2015). Because of this, previous studies are likely to have failed to account for NX-2 genotypes and to have overestimated the proportion of 3ADON strains in the total *F. graminearum* population.

To date, all strains with the NX-2 *Tri1* allele have been found in a genetic background with a 3ADON-like major trichothecene gene cluster (Kelly et al. 2016; Liang et al. 2014). Theoretically, the equivalent 15ADON molecule, known as NX-4, may exist but has never been identified. It is therefore likely that strains previously identified as 3ADON by PCR directed solely to the trichothecene gene cluster may actually be the NX-2 chemotype. A PCR-RFLP based assay directed toward *Tri1* now has been developed to distinguish the NX-2 and 3ADON genotype (Liang et al. 2014). Using a large set of *F. graminearum* strains from Willsboro, in Essex County, New York, USA, which had been previously characterized as possessing an unusually high frequency (47.6%) of strains assigned a 3ADON genotype, we characterized the abundance of the NX-2 strains present among a background population rich in 3ADON producers. We found that a large proportion of those strains previously characterized as being the 3ADON genotype were actually the NX-2 genotype as determined by *Tri1* allele genotyping and by biochemical confirmation.

Experimental procedure and results

The strains used in this study were previously described at Cornell University Willsboro Research Farm in Willsboro, NY, and were chosen for this study because the collection contained an unusually high frequency of strains (47.6%) assigned a 3ADON trichothecene gene cluster genotype (Kuhnem et al. 2015b). Because NX-2 producing strains would not have been detected by previously employed genotyping methods and since NX-2 strains also would have a 3ADON trichothecene gene cluster genotype, we chose to re-examine this collection for the presence of NX-2 producing strains. We were able to revive 133 of 152 strains previously assigned a 3ADON genotype from this collection (Table 1). These included all 43 strains from wheat (100%), and 29 of 30 from maize stubble (96.7%), but only 49 of 58 (84.5%) from air and 12 of 21 (57.1%) from maize ears. An additional isolate from maize in western New York, GZ830, shown to be highly virulent on wheat and maize but which produced no DON,

Table 1 Trichothecene genotype composition of *F. graminearum* samples isolated from different substrates in Willsboro, NY in 2013

Substrate	Total[a]	15ADON[b]	"3ADON[b]"	"3ADON[c]" recovered	3ADON[d]	NX-2[d]
Wheat spike	98	55 (56.1%)	43 (43.9%)	43 (100%)	28 (28.6%)	15 (15.3%)
Air	101	43 (42.6%)	58 (57.4%)	49 (84.5%)	29 (34.0%)[e]	20 (23.4%)
Maize stubble	65	35 (53.8%)	30 (46.2%)	29 (96.7%)	16 (25.5%)	13 (20.7%)
Maize ear	55	34 (61.8%)	21 (38.1%)	12 (57.1%)	4 (12.7%)	8 (25.5%)
Total	319	167 (52.4%)	152 (47.6%)	133 (87.5%)	77 (27.6%)	56 (20.0%)

[a] Total number of strains examined in this study from each substrate

[b] Number and proportion of 15ADON and "3ADON" genotypes for strains previously reported in (Kuhnem et al. 2015b). "3ADON" indicates that the reported class actually contains both NX-2 and authentic 3ADON strains

[c] Number and proportion of "3ADON" strains from Kuhnem et al. (2015b), recovered for this study

[d] Number and proportion of "3ADON" strains having the authentic 3ADON or NX-2 genotypes

[e] Underlined values are proportions corrected to account for strains missing from the original collection

3ADON, or 15ADON in culture (Kuhnem et al. 2015b), was also included in this study. DNA was extracted following (Gale et al. 2007). The *Tri1* allele was assayed for NX-2 genotypes following (Liang et al. 2014), and chemotype diagnostic trichothecene biosynthetic gene cluster polymorphisms were characterized as described in (Ward et al. 2002). Biochemical trichothecene profiles were generated for eight of the 56 strains assigned the NX-2 chemotype by genetic testing. Biochemical detection of NX-2 by GC-MS was conducted as described in (Varga et al. 2015).

In total, 56 of the 133 recovered strains (42.1%) had a *Tri1* allele genotype consistent with NX-2 producing strains. To confirm that these were authentic NX-2 producers, seven of the 56 strains (designated GZ3399, GZ3402, GZ3668, GZ3843, GZ4302, GZ4326, and GZ4347) were tested by GC-MS for the presence of NX-2 when grown in culture under trichothecene-inducing conditions. All strains accumulated NX-2, but no detectable 3ADON, in culture.

NX-2 genotype strains were found from all sampled origins (Table 1). Because all strains from wheat heads were recovered from storage, we were able to directly calculate that this collection of 98 strains consisted of 56.1% 15ADON strains, 28.6% 3ADON strains and 15.3% NX-2 strains based on *Tri1* allele genotyping. This proportion of NX-2 genotype strains is 7- to 14-fold higher than levels previously observed in the northcentral U.S. and southern Canada (2.2%) or in Connecticut (1.1%) (Kelly et al. 2016).

Strains derived from origins other than wheat may represent an even higher proportion of NX-2 genotype strains. However, because we were unable to recover all of the strains from the original collection, the frequencies of each chemotype could not be directly calculated. Rather, the frequency of NX-2 genotype strains among the collection was inferred by proportionally correcting for missing strains. (The inferred corrected percentage of NX-2 strains per substrate is given in Table 1 as an underlined value). This assumes NX-2 and 3ADON strains had an equal likelihood of being recovered from the 3ADON collection of strains. The correction would create little error for the maize stubble collection where 29 of 30 samples were recovered but could introduce considerable error for the maize ear collection where only 12 of 21 were recovered. The proportion of NX-2 genotype strains derived from other origins ranged from 20.7% for the maize stubble collection to 25.5% for the maize ear collection.

To determine if the NX-2 *Tri1* allele could be found in a genetic background containing a 15ADON trichothecene gene cluster, the 55 15ADON strains from wheat spikes in the Willsboro study were also examined. Because *Tri1* and the trichothecene gene cluster are genetically unlinked, recombinant genotypes should be found based on the high frequency of the NX-2 Tri1 allele if random mating was occurring. Such recombinants would be expected to produce the compound NX-4, which is identical to 15ADON but lacking the keto group at the 8 position carbon like NX-2 (Varga et al. 2015). In fact, two strains (GZ3700 and GZ3704) were determined to have an NX-2-like *Tri1* allele. However, re-examination of the trichothecene gene cluster of these strains indicated that they had a 3ADON-like genotype

and therefore represented two additional NX-2 strains. We do not know if this discrepancy was due to previous error in genotype assignment of the trichothecene gene cluster or a mix-up in strains due to handling between isolation and final analysis. Nevertheless, with the two additional NX-2 strains identified in place of two 15ADON strains, it is possible that the frequency of NX-2 genotypes in the Willsboro collection was even higher than indicated above.

Strain GZ830 from maize in western New York also was identified in this study to be the NX-2 genotype. This solves the mystery of why this strain, which had previously been shown to be highly virulent on wheat and maize, produced no detectable DON or ADON forms in infected grain (Kuhnem et al. 2015a).

Discussion

In this study, we tested the prevalence of the NX-2 genotype in a *F. graminearum* population that had been previously characterized as highly enriched in 3ADON strains. We found that an unexpectedly high proportion of these strains were of the NX-2 and not the 3ADON genotype. NX-2 genotype strains were isolated from diverse origins, but all possessed a 3ADON major trichothecene gene cluster. Conversely, none of the strains possessing a 15ADON gene cluster tested positive for the ability to encode NX-4.

A recent survey of a worldwide collection of over 2500 *F. graminearum* strains found that the NX-2 genoype is endemic to North America and more specifically found only in the northeastern and northcentral U.S. and southcentral and southeastern Canada (Kelly et al. 2016). NX-2 strains have been isolated from wheat, maize, barley, oat (Kelly et al. 2015; Liang et al. 2014; Kelly et al. 2016) and airborne environmental samples (this report). When found previously, the frequency of NX-2 strains varied from 1.1% (maize, Connecticut) to 6% (barley, Québec) (Kelly et al. 2016).

With this report, we show that a local population of *F. graminearum* contains a much higher frequency of NX-2 isolates than previously observed. In fact, the local collection of 319 isolates from Willsboro NY has more NX-2 genotype strains than the total worldwide collection of 2515 strains previously reported by Kelly et al. (2016). The Willsboro samples more than double the number of known NX-2 isolates identified from nature to 99 (previously only 41 were known). It's currently unclear why this site has such a high frequency of potential NX-2 producers. The Willsboro Farm falls within a distinctive agricultural landscape wedged between the Adirondack Mountains and Lake Champlain. Regional environmental conditions and differences in alternative hosts have been suggested as influencing *F. graminearum* chemotype composition (Liang et al. 2015) but direct experimental evidence is lacking.

Equally puzzling is why no NX-4 genotype strains (those having a NX-2-like *Tri1* allele and a 15ADON-like trichothecene gene cluster) were identified in the Willsboro samples. Previous studies, however, have suggested that there may be barriers to the formation of such recombinant genotypes that are associated with distinct genetic populations of *F. graminearum* (Liang et al. 2014). The distribution of NX-2 strains is similar to that of the emergent NA2 population (previously called the UMW 3ADON population) of *F. graminearum* that consists largely of 3ADON genotype strains and also marginally overlaps the northern range of the NA1 population (previously called the MW 15ADON population) that predominates in North America and consists largely of 15ADON genotype strains (Gale et al. 2007; Ward et al. 2008; Schmale et al. 2011). Preliminary population genetic analysis, based on a limited number of NX-2 strains, suggests that the NX-2 genotype may belong to yet another genetic population distinct from NA1 and NA2 (Liang et al. 2014; Kelly et al. 2015, 2016). Further work is needed to accurately place NX-2 strains within the current population structure of *F. graminearum* in North America and to better understand barriers to unfettered gene flow within the species.

Although previous in-vitro work has shown equivalent toxicities between 3ADON and NX-2 as well as their equivalent deacetylated versions, DON and NX-3 (Varga et al. 2015), the consequences of fungal chemotype on plant disease is largely unexplored. However, because chemotype differences could potentially influence fungal virulence and patterns of host specificity (Maier et al. 2006), characterizing the functional implications of trichothecene variants as well as more comprehensive studies of the distribution and diversity of these toxigenic strains has the potential to inform both disease management and plant breeding. Because the existence of NX-2 producing strains was so recently discovered, little work has been conducted to determine whether plants developed for resistance to DON producing strains of *F. graminearum* may also be resistant to NX-2 producing strains. As local populations with

substantial proportions of NX-2 genotype strains are discovered, it will be prudent to assure that these strains are included in efforts to discover and incorporate plant disease resistance into cultivated crops.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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