The 3ADON Population of *Fusarium graminearum* Found in North Dakota Is More Aggressive and Produces a Higher Level of DON than the Prevalent 15ADON Population in Spring Wheat

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Accepted for publication 9 May 2010.

ABSTRACT

Puri, K. D., and Zhong, S. 2010. The 3ADON population of *Fusarium graminearum* found in North Dakota is more aggressive and produces a higher level of DON than the prevalent 15ADON population in spring wheat. *Phytopathology* 100:1007-1014.

Fusarium head blight (FHB) is primarily caused by *Fusarium graminearum* in North America. Isolates of *F. graminearum* can be identified as one of three chemotypes: 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON), and nivalenol (NIV). In this study, we characterized *F. graminearum* isolates collected in 1980 to 2000 (old collection) and in 2008 (new collection) from North Dakota and found a 15-fold increase of 3ADON isolates in the new collection. Evaluation of randomly selected 3ADON isolates and 15ADON isolates on three spring wheat genotypes (Grandin, Steele-ND, and ND 2710) by single-floret

inoculation indicated that the 3ADON population caused a higher disease severity and produced more DON at a significant level than the 15ADON population on Grandin (susceptible to FHB) and ND 2710 (with FHB resistance from Sumai 3). However, no significant differences in disease severity and DON production were observed between the two populations on Steele-ND (with moderate resistance from *Triticum dicoccoides*). The 3ADON isolates also exhibited a higher DON production in rice culture and produced more spores on agar media than the 15ADON isolates, suggesting a fitness advantage of the newly emerging 3ADON population over the prevalent 15ADON population. Population genetic analyses using DNA markers revealed a significant genetic differentiation between the two populations. The information obtained in this study could have an impact on development of FHB-resistant wheat cultivars and disease management.

Fusarium head blight (FHB) or head scab is a devastating disease of wheat (*Triticum aestivum* L) and barley (*Hordeum vulgare* L) in North America (15,21,25,53) and many other regions of the world (17,37). The disease not only causes significant yield losses but also reduces the quality of harvested grains due to contamination of mycotoxins produced by the *Fusarium* pathogens (5,43). Consumption of mycotoxin-contaminated grains and their products poses acute or chronic health hazards to human and livestock (8,32).

Several *Fusarium* spp. can cause FHB in barley and wheat but *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch) is the primary causal agent of the disease in North America. The fungal pathogen belongs to the *F. graminearum* species complex, which has been separated into at least 11 distinct phylogenetic species (35,45). The *F. graminearum* complex and the other five closely related species produce various B-trichothecene metabolites, including deoxynivalenol (DON) and its derivatives (3-acetyl deoxynivalenol [3ADON] and 15-acetyl deoxynivalenol [15ADON]) and nivalenol (NIV) (34). Based on the profile of trichothecenes produced, *F. graminearum* isolates can be grouped into one of three chemotypes (i.e., 3ADON, 15ADON, and NIV) (30,35). A 3ADON chemotype produces DON and primarily 3ADON while a 15ADON chemotype generates DON and primarily 15ADON. Isolates with a NIV chemotype produce NIV and its acetylated derivatives. Previous studies indicated that isolates with a 15ADON chemotype in the *F. graminearum* complex were predominant and largely responsi-

ble for FHB epidemics in North America (1,30). Zeller et al. (55,56) analyzed and compared populations of *F. graminearum* from seven states of the central and eastern United States and found a low genetic differentiation among them. They suggested that these local populations represent a subset of a larger pan-mictic population in North America (56). However, several recent studies indicated that genetically differentiated populations of *F. graminearum* have emerged in Canada and the United States. In a global molecular surveillance of FHB pathogens, Starkey et al. (45) identified six *F. graminearum* isolates with a NIV or 3ADON chemotype in Louisiana. Gale et al. (13) identified highly divergent populations of *F. graminearum* in North Dakota and Minnesota where 3ADON isolates were identified, which showed a significant genetic differentiation from the 15ADON populations. More recently, Ward et al. (52) and Guo et al. (19) reported a significant increase of the *F. graminearum* isolates with the 3ADON chemotype in Canada. The newly emerging 3ADON population appears to be more aggressive than the 15ADON population based on growth rate and DON production in vitro (52). However, little information is available on the aggressiveness and DON production of the 3ADON population in spring wheat cultivars with different sources of FHB resistance. The objective of this study was to test the hypothesis that the 3ADON population newly sampled from spring wheat in North Dakota is more aggressive in disease development and DON production compared with the prevalent 15ADON population. We characterized *F. graminearum* isolates collected in 1980 to 2000 and those collected during 2008 from North Dakota spring wheat by multiplex polymerase chain reaction (PCR) chemotyping, greenhouse inoculation on three wheat genotypes with different levels of FHB resistance, amplified fragment length polymorphism (AFLP), and variable number of tandem repeat (VNTR) analyses.

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doi:10.1094/PHYTO-12-09-0332

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MATERIALS AND METHODS

Fungal isolation and identification. A total collection of 165 isolates of *F. graminearum*, including 120 collected in 1980 to 2000, 2 collected in 2004, and 43 collected in 2008 from different counties of North Dakota, was used in this study. These fungal isolates were isolated from symptomatic wheat spikes collected from farmers' fields. Kernels showing FHB symptoms were surface sterilized with 6% sodium hypo-chloride for 2 to 3 min, rinsed two to three times with sterile distilled water, and kept on three layers of moist blotting paper. Visible fungal mycelia grown from kernel surfaces were transferred into half-strength (1/2) potato dextrose agar (PDA) (100 g of potato, 10 g of dextrose, and 7.5 g of bacto-agar per liter of water). After 5 to 6 days of growth, spore mass was picked with an inoculation loop and streaked onto a new 1/2-PDA plate. The individual single spores were allowed to germinate for 24 h. After that, a single-spore colony was picked and transferred into a new 1/2-PDA plate. After 7 days of growth, the single-spore cultures were stored in 20% glycerol and kept at -80°C until needed. Morphological characters as well as the *F. graminearum*-specific PCR primer sets, UBC85F410/UBC85R410 (42) and Fg16F/Fg16R (33), were used to confirm the identity of all isolates used in the study. To further identify the isolates in the *F. graminearum* complex to the phylogenetic species level (35), portions of the reductase and histone H3 genes were amplified by PCR from each of the isolates and sequenced according to the methods previously described (34,35).

DNA extraction and PCR-based chemotyping. DNA was extracted from mycelia of the 6- and 7-day-old single-spore cultures grown over cellophane on 1/2-PDA using the FastDNA Kit and FastPrep instrument (MP Biomedicals, Irvine, CA). For chemotype determination, the trichothecene primer 3CON (5-TGGC AAAGACTGGTTCAC-3) and the three chemotype-specific primers 3NA (5-GTGACAGAAATATACGAGC-3), 3D15A (5-ACTGACCCAAGCTGCCATC-3), and 3D3A (5-CGATTGGC TAACACATG-3) were used for PCR amplification with the conditions described by Ward et al. (51) and Starkey et al. (45). These primer combinations amplified fragments of 840, 610, or 243 bp that correspond to the NIV, 15ADON, or 3ADON chemotypes, respectively (51).

Aggressiveness evaluation. A greenhouse experiment was conducted to test the aggressiveness of 25 randomly selected isolates of *F. graminearum* (12 15ADON and 13 3ADON isolates) (Table 1) on the spring wheat genotypes Grandin, Steele-ND, and ND2710. Grandin is a spring wheat cultivar that is highly susceptible to FHB. Steele-ND exhibits a moderate level of resistance to FHB, which is thought to be derived from *T. dicoccoides* (27). ND2710 is a spring wheat line with a high level of FHB resistance derived from Sumai 3 (11). A randomized complete block design (RCBD) was used with three replications and all isolates were randomized within each replication. For each wheat genotype, hill plots per replication were planted on a bed filled with sterilized topsoil, with a 6-in. space between plots and 8 to 10 seeds/plot. The plants were grown at $23 \pm 2^{\circ}\text{C}$ with 16 h of supplemental lights until anthesis. Irrigation was done daily and plants were fertilized with Multicote 4 containing N:P:K (14:14:16) and minor nutrients (Heifa Biochemical, Israel).

To prepare spores for inoculation, each of the isolates was grown on mung bean agar (MBA) (10) plates at room temperature with a combination of fluorescent and near-UV light at a 12-h light and dark cycle for 10 to 12 days. Then conidia were harvested from the agar plates by adding sterilized distilled water and scraping the agar surface with a rubber policeman, then filtered through two layers of cheesecloth and quantified with a hemacytometer. Final inoculum at 1×10^5 macroconidia/ml in water was used to inoculate wheat spikes using the single-floret injection method (41). The central floret of individual spikes at anthesis was inoculated with 10 μl of the inoculum using a micro-

pipette. Inoculated heads were misted with the overhead misting nozzles for 30 s at 30-min intervals to maintain high humidity. Disease rating was done at 21 days after inoculation (dai) using the pictorial scale developed by Stack and McMullen (44). The disease severity (DS) for each inoculated spike was measured based on the number of spikelets infected: no infection = 0%, one spikelet infected = 7%, two = 14%, three = 21%, four = 33%, one-half of the spike = 50%, two-thirds of the spike = 67%, three-quarters of the spike = 75%, and the whole spike or dead rachis = 100%. At least eight spikes were inoculated and rated in each replication (hill plot) for each wheat genotype. The average DS was calculated according to Stack and McMullen (44).

Analysis of trichothecenes from grains and rice cultures. For evaluation of trichothecene accumulation in grains, the inoculated heads of each cultivar from each replication were harvested and threshed. The grains were ground to a fine powder using a coffee grinder and sent to the Veterinary Diagnostic Laboratory, North Dakota State University, for mycotoxin analyses. The amount of DON, its derivatives (3ADON and 15ADON), and NIV was quantified using the gas chromatography-mass spectrometry (GC-MS) method (47).

Mycotoxin production in rice cultures was measured according to Burlakoti et al. (6) and Walker et al. (49) with some modifications. Rice grains (30 g) were soaked in deionized distilled water overnight (10 h) in a 250-ml Erlenmeyer flask. Excess water was drained from the flask. After autoclaving, the rice grains were inoculated with three plugs of agar containing growing mycelia from a 4- to 5-day-old culture on PDA. Each flask was considered as one replication and three flasks for each isolate were prepared. To facilitate equal distribution of fungal growth, the culture was stirred with a sterile glass rod on the second, fourth, and sixth days. Then, the cultures were grown in the dark for 30 days at $23 \pm 1^{\circ}\text{C}$. Each rice culture was transferred into a 50-ml centrifuge tube and stored at -80°C overnight and lyophilized for 6 days at -40°C . Then, the rice cultures were ground with a coffee grinder and 5 g of each sample was analyzed for mycotoxins using the same method as used for the grain samples.

Growth rate and sporulation test. The growth rate and sporulation of isolates were evaluated in vitro on MBA plates in three replications. A small agar plug (5 mm in diameter) of each isolate was placed in the center of a 9-cm MBA plate and incubated the same way as that for inoculum preparation. Radial growth was measured every 24 h until mycelia completely covered the plate surface. After 7 days of growth, conidia were harvested by adding 1 ml of distilled water and scraping the surface of the agar with a rubber policeman. The spores were counted under a compound microscope using a hemacytometer.

AFLP analysis. AFLP analysis was performed according to Vos et al. (48) with some modifications. DNA templates for pre-selective amplification were prepared using the AFLP Core Reagent Kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's protocol. Preamplification reactions contained 1:2 diluted DNA templates (≈ 62.5 ng of DNA), 36 ng of each *EcoRI* primer (E-A) and *MseI* primer (M-C), 0.2 mM dNTPs, 1 $\times$ buffer, 1.5 mM MgCl_2 , and 1 unit of *Taq* DNA polymerase and was preformed for 20 cycles of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s. Five sets of primer combinations (E-AC+M-CG, E-AT+M-CC, E-AA+M-CC, E-AA+M-CT, and E-AA+M-AT) were used in the selective amplification, with the E- primers labeled with the fluorescent dye IRD 700 (Li-COR, Lincoln, NE). The selective amplification conditions were the same as described by Vos et al. (48). After selective amplification, the PCR reaction was mixed with 10 μl of Formamide loading dye, heated at 95°C for 3 min, immediately chilled on ice, and loaded (0.5 μl) on a polyacrylamide gel in the Li-COR system (Li-COR). The gel was prepared by using 20 ml of Rapid Gel-XL 6% Liquid Acrylamide (USB Corporation, Cleveland), 15 μl of tetramethylethylenediamine, and freshly prepared 10% ammonium persulfate. AFLP

fragments of 90 to 600 bp were scored manually with reference to a 50-bp ladder (Li-COR) and those fragments absent in at least one isolate but present in other isolates were considered to be polymorphic. The presence and absence of fragments were scored as one and zero, respectively.

VNTR analysis. Nine VNTR markers (*HK913*, *HK917*, *HK957*, *HK965*, *HK967*, *HK977*, *HK1059*, *HK1073*, and *HK1043*) developed by Suga et al. (46) and one additional VNTR marker (*HK1003*) reported by Gale et al. (12) were used to analyze the same set of isolates used in the AFLP analysis. To detect VNTR markers on the Li-COR system (Li-COR), forward primers were synthesized with the M13 primer sequence (CACGACGTT GTAAAACGAC) added at the 5' end and the M13 primer labeled with IRD-700 or -800 (Eurofins MWG Operon, AL) was included in the PCR reactions. Each PCR reaction contained a total volume of 10 µl consisting of 1× buffer, 200 µM of dNTP, 1.0 µM of each primer (forward, reverse, and IRD-700/800-labeled M13), 1 unit of *Taq* polymerase, and genomic DNA at 25 to 30 ng/µl. PCR amplification was performed using a PTC-100 thermal cycler (MJ Research). Amplification was completed with initial denaturation at 94°C for 3 min; followed by 3 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; an additional 25 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 45 s; and final extension at 72°C

for 5 min. PCR products were loaded on the Li-COR system (Li-COR) following the same procedure used for detecting AFLP markers, as described above. VNTR alleles were scored manually with reference to a 50-bp ladder (Li-COR). For each VNTR locus, amplicons with the same size were considered the same alleles.

Statistical analyses. Analysis of variance (ANOVA) for DS and mycotoxin accumulation was performed on arcsine and log-transformed data, respectively (16). ANOVA, least significance difference (LSD) for mean separation, and Pearson Correlation Coefficients and *t* test for population comparison were performed using SAS (version 9.1.3; SAS Institute, Cary, NC). Standard deviation and standard error in DS and mycotoxin accumulation among isolates were calculated using Microsoft Excel 2003.

Genetic distance (*D*), Nei's unbiased gene diversity (*H*) (31), and gene flow (*N_m*) (24) were analyzed using POPGENE, version 1.32 (54). The software Multilocus 1.3 was used to calculate the genotype diversity (*GD*) as $(n/n - 1) (1 - \sum p_i^2)$, where p_i is the frequency of the *i*th genotype and *n* is the number of individuals sampled (2). Then, the index of linkage disequilibrium (*LD*) was calculated to test nonrandom association of alleles at different gene loci based on the index of association (*I_A*). GenAlEx 6 was used for pairwise population differentiation (*F_{st}*), gene flow (*N_m*), and analysis of molecular variance (AMOVA) (39).

TABLE 1. Chemotype, aggressiveness based on disease severity, and trichothecenes of the *Fusarium graminearum* isolates used in the study[†]

Isolate	Year [‡]	Origin	Chemotype [‡]	DS (%) [‡]	Trichothecenes in rice culture (ppm) [§]				Trichothecenes in grains (ppm) [§]		
					DON	3ADON	15ADON	NIV	DON	3ADON	15ADON
Fg08-001	2008	Foster, ND	3ADON	55.6 ± 5.4 a	253.8 ± 27.6	142.9 ± 18.3	*	*	22.5 ± 8.0	1.2	—
Fg08-002	2008	Barnes, ND	3ADON	23.2 ± 5.8 ik	25.7 ± 7.3	3.4 ± 1.4	—	—	5.1 ± 1.4	—	—
Fg08-003	2008	Steele, ND	3ADON	44.8 ± 3.9 af	305.4 ± 44.3	182.6 ± 33.8	*	*	24.0 ± 4.0	0.6	—
Fg08-004	2008	Barnes, ND	3ADON	45.7 ± 4.6 ae	305.3 ± 59.5	172.8 ± 19.9	2.1 ± 0.1	*	16.9 ± 10.7	0.9	—
Fg08-005	2008	Foster, ND	3ADON	36.5 ± 3.5 dh	221.8 ± 1.7	94.6 ± 11.2	*	*	15.7 ± 5.1	0.9	...
Fg08-006	2008	Steele, ND	3ADON	51.7 ± 6.8 ab	298.3 ± 14.5	210.3 ± 15.8	2.1 ± 0.1	*	21.7 ± 5.1	0.6	—
Fg08-007	2008	Foster, ND	15ADON	31.8 ± 0.2 fi	188.8 ± 33.5	*	19.0 ± 6.7	*	7.9 ± 2.2	—	0.6
Fg08-008	2008	Steele, ND	3ADON	46.0 ± 7.4 af	300.4 ± 7.3	228.6 ± 29.7	*	*	19.3 ± 3.1	0.7	—
Fg08-009	2008	Foster, ND	3ADON	51.4 ± 10.6 ac	398.5 ± 81.8	258.2 ± 38.1	2.8 ± 0.8	*	20.7 ± 9.0	1.7	—
Fg08-010	2008	Barnes, ND	3ADON	42.7 ± 3.4 ag	282.5 ± 47.2	235.5 ± 36.7	2.1 ± 0.1	2.2	16.9 ± 6.8	—	—
Fg08-011	2008	Foster, ND	3ADON	47.9 ± 3.3 ad	320.9 ± 71.1	252.7 ± 36.2	2.3 ± 0.3	*	21.5 ± 7.5	0.7	—
Fg08-012	2008	Steele, ND	3ADON	45.7 ± 5.9 ae	273.5 ± 16.4	174.6 ± 19.0	*	*	27.6 ± 5.0	1.1	—
Fg08-013	2008	Steele, ND	15ADON	31.6 ± 2.4 fi	158.9 ± 23.7	*	3.5 ± 0.4	*	3.4 ± 1.2	—	—
Fg08-018	2008	Steele, ND	15ADON	39.2 ± 3.2 bh	396.2 ± 40.9	2.1 ± 0.1	10.1 ± 3.1	*	6.8 ± 2.5	—	—
Fg08-019 ^z	2008	Foster, ND	3ADON	...	...	...	...	...	...	...	...
Fg08-020 ^z	2008	Barnes, ND	3ADON	...	...	...	...	...	...	...	...
Fg08-021 ^z	2008	Barnes, ND	3ADON	...	...	...	...	...	...	...	...
R010	1983	North Dakota	15ADON	38.0 ± 2.2 dh	398.1 ± 77.7	*	11.3 ± 1.9	*	14.9 ± 4.0	—	0.6
R366	1981	North Dakota	15ADON	23.2 ± 2.6 ik	1.3 ± 0.7	—	—	—	2.8 ± 1.3	...	—
R458 ^z	1981	North Dakota	3ADON	...	...	...	...	...	...	...	...
R459	1981	North Dakota	3ADON	33.4 ± 2.9 ei	4.4 ± 0.2	—	*	...	2.3 ± 1.4	—	—
R465	1981	North Dakota	15ADON	13.5 ± 3.6 k	*	*	*	*	2.7 ± 0.9	—	—
R1171	1991	Cavalier, ND	15ADON	35.4 ± 7.0 di	195.1 ± 49.0	2.2 ± 0.2	12.0 ± 6.0	*	10.5 ± 3.7	—	0.6
R1215 ^z	1993	North Dakota	15ADON	...	...	...	...	...	...	...	...
R1247	1993	North Dakota	15ADON	16.3 ± 5.2 jk	*	*	*	*	1.5 ± 0.3	—	—
R1265 ^z	1994	North Dakota	15ADON	...	...	...	...	...	...	...	...
R1310	1995	Benson, ND	15ADON	34.3 ± 1.1 di	145.9 ± 66.6	2.5 ± 0.7	17.5 ± 7.0	*	8.7 ± 1.0	—	—
R1318	1995	Cass, ND	15ADON	38.2 ± 6.2 ch	136.8 ± 8.6	*	11.2 ± 1.2	*	12.1 ± 4.9	—	—
R1390	1990	North Dakota	15ADON	26.4 ± 2.7 ij	107.8 ± 20.4	*	3.8 ± 1.2	*	6.9 ± 2.7	—	—
R1391	1990	North Dakota	3ADON	29.6 ± 5.0 gi	144.1 ± 19.0	61.6 ± 7.1	*	*	11.1 ± 3.0	—	—
R1628 ^z	2001	Mountrail, ND	15ADON	...	...	...	...	...	...	...	...
R1694 ^z	2004	Cass, ND	15ADON	...	...	...	...	...	...	...	...
R1700	2004	Cass, ND	15ADON	45.8 ± 5.4 ae	285.0 ± 14.4	*	5.3 ± 1.6	*	12.8 ± 5.1	—	0.9

[†] DON = deoxynivalenol, 3ADON = 3-acetyl-deoxynivalenol, 15ADON = 15-acetyl-deoxynivalenol, and NIV = nivalenol; ... indicates the information is unknown.

[‡] Year of collection.

[§] Chemotype was determined by the multiplex polymerase chain reaction assay of Ward et al. (51).

[¶] Disease severity (DS) was recorded from Grandin, Steele-ND, and ND 2710 at 21 days after inoculation and the averaged DS was calculated across the three wheat genotypes for each isolate. Least significant difference analysis was done with the Arcsine transformed value of DS. DS values indicated with the same lowercase letters were not significantly different at $\alpha = 0.05$.

[§] Trichothecenes in rice culture were measured and averaged from three replications; ppm = part per million (mg/kg of samples), * = not detected at <2 ppm, — = not detected at <0.5 ppm, ± = standard error of mean.

[§] Trichothecenes in grains were obtained from wheat heads inoculated in the greenhouse and averaged from the three wheat genotypes; ppm = part per million (mg/kg of samples), — = not detected at <0.5 ppm, ± = standard error of mean.

^z These isolates were only included in population genetic analyses.

RESULTS

***Fusarium* spp. identification and trichothecene chemotyping.** The primers UBC85F410 and UBC85R410 generated a *F. graminearum*-complex-specific 332-bp amplicon, which was observed for all isolates analyzed except for R709. Phylogenetic analysis of the reductase and histone H3 gene sequences indicated that R709 is *F. culmorum* and all other isolates belong to the *F. graminearum* sensu (s.) strict (str.) (data not shown).

Among the 120 isolates collected in 1980 to 2000 in North Dakota, 93% were of the 15ADON chemotype and only a few isolates (3 and 4%) were of the 3ADON or NIV chemotypes, respectively (Fig. 1A). In contrast, 44% of the 43 isolates from the new collection (2008) were of the 3ADON chemotype, while 56% of the isolates were of the 15ADON chemotype and no NIV isolates were found (Fig. 1B).

Aggressiveness evaluation on spring wheat genotypes. The 25 isolates of *F. graminearum* s. str. evaluated on the three spring wheat genotypes (Grandin, Steele-ND, and ND2710) varied in aggressiveness. ANOVA indicated that interaction between fungal isolate and wheat genotype was not significant but DS was significantly different among isolates or wheat genotypes (Table 2). The average DS caused by individual isolates pooled based on wheat genotype ranged from 13.5 ± 3.6 to $55.6 \pm 5.4\%$. Isolate Fg08-001 had the highest DS but was not significantly different from some other isolates (Fg08-003, Fg08-004, Fg08-006, Fg08-008, Fg08-009, Fg08-010, Fg08-011, Fg08-012, and R1700). The majority of the 3ADON isolates was highly aggressive, causing a higher level of disease than the 15ADON isolates, except for R1700 (Table 1).

Among the three wheat genotypes tested, ND2710 showed the lowest DS (DS = 21.2%) while no significant differences were observed between Grandin (DS = 46.4%) and Steele-ND (DS = 42.4%). The two populations (3ADON and 15ADON) interacted differently with the wheat genotypes, with the 3ADON population causing a significantly higher DS than the 15ADON population on the susceptible Grandin ($t = 4.4$, $P < 0.0001$) and the moderately resistant ND2710 ($t = 2.7$, $P = 0.0087$) (Fig. 2). However, no significant difference in DS was detected on Steele-ND between the two populations ($t = 1.9$, $P = 0.065$) (Fig. 2).

Trichothecene production in rice culture and grains. The total amount of DON, 15ADON, and 3ADON produced by individual isolates in rice cultures varied, ranging from no detection to 398.5 ± 81.5 ppm (standard error) (Table 1). Two 15ADON isolates (R465 and R1247) had DON production below the detection level. All 3ADON isolates, except for Fg08-002 and

R459, produced a large amount of 3ADON (61.6 to 258.2 ppm) but the amount of 15ADON produced by the 15ADON isolates was relatively low (maximum of 19 ppm) (Table 1). Grouping of the isolates based on the chemotypes showed that the 3ADON population produced 1.5 times more DON and 86 times higher 3ADON but 6 times less 15ADON than the 15ADON population in rice cultures (Table 3).

TABLE 2. Analysis of variance of disease severity (DS) on the three wheat genotypes (Grandin, Steele-ND, and ND 2710) inoculated by 25 isolates of *Fusarium graminearum*

Source of variation	df	MS ^y	F value ^z
Fungal isolate	24	447.5	5.47**
Wheat genotype	2	5,618.2	68.67**
Fungal isolate × wheat genotype	48	83.5	1.02 ns

^y Arcsine transformation of DS was made for analysis. Mean square (MS) was derived from the type III sum of squares.

^z Asterisks (**) = highly significant ($P < 0.0001$) and ns = not significant ($P = 0.45$).

Fig. 2. Disease severity (DS) on each of the three wheat genotypes (Grandin, Steele-ND, and ND 2710) averaged based on chemotype populations (3-acetyl-deoxynivalenol [3ADON] or 15-acetyl-deoxynivalenol [15ADON]). The 3ADON population caused a higher DS on Grandin and ND 2710 than the 15ADON population at a highly significant ($P < 0.0001$) and significant level ($P = 0.0087$), respectively. However, no significant difference in DS was detected on Steele-ND between the two populations ($P = 0.065$).

Fig. 1. Frequency distribution of chemotypes among the 120 *Fusarium graminearum* isolates collected during 1980–2000 (A) and the 43 isolates collected in 2008 (B) from different locations of North Dakota.

The DON levels in grain harvested from the inoculated spikes were relatively low compared with those produced in rice cultures, ranging from 1.5 ± 0.3 to 27.6 ± 5.0 ppm (Table 1). The mean DON accumulated in grains from the susceptible cv. Grandin (18.8 ppm) was significantly higher than that accumulated in those from ND2710 (8.17 ppm) and Steele-ND (10.47 ppm). Although Steele-ND and ND2710 showed significant differences in DS, the DON accumulation was similar between these two wheat genotypes, suggesting that different mechanisms may operate for resistance to FHB severity and DON accumulation. Comparison of the two populations (3ADON and 15ADON) on individual wheat genotypes indicated that the 3ADON population produced a significantly higher DON than the 15ADON population on Grandin ($P = 0.0006$) and ND 2710 ($P = 0.0016$) (Fig. 3). Interestingly, the two populations did not show a significant difference in DON accumulation on Steele-ND ($P = 0.1057$) (Fig. 3).

Growth rate and sporulation. No significant differences in growth rates were observed between isolates of the 3ADON population and those of the 15ADON population (data not shown); however, the 3ADON isolates produced a significantly ($P = 0.05$) higher number of macroconidia (4.4 ± 0.60 million conidia/ml) on MBA compared with the 15ADON isolates (3.3 ± 0.74 million conidia/ml).

Correlation between trichothecene production and DS. A highly significant positive correlation existed between DS and DON production in both rice culture ($R = 0.58$, $P \leq 0.01$) and grains ($R = 0.85$, $P \leq 0.01$) (Fig. 4A and D). A significant positive correlation was also identified between DS and 3ADON ($R = 0.57$, $P \leq 0.01$) produced in rice culture (Fig. 4B). However, no significant correlation was found between DS and 15ADON amount ($R = -0.05$, $P = 0.64$) (Fig. 4C).

Population genetic analyses. In all, 17 3ADON isolates and 16 15ADON isolates (Table 1) were subjected to AFLP analysis. The five primer pairs (E-AC+M-CG, E-AT+M-CC, E-AA+M-CC, E-AA+M-CT, and E-AA+M-AT) generated 40, 39, 23, 38, and 32 polymorphic fragments, respectively. Among the 172 AFLP loci scored, 70.9% were polymorphic among the 15ADON isolates compared with 64.5% among the 3ADON isolates. Both 15ADON and 3ADON populations showed a GD value of 1, indicating that all isolates had a unique genotype. The gene diversity (H) was 0.189 for the 15ADON population and 0.166 for the 3ADON population. The LD value was 0.057 ($P < 0.001$) for the 15ADON population and 0.075 ($P < 0.001$) for the 3ADON population. Genetic differentiation between the two populations was significant ($F_{st} = 0.065$, $P = 0.001$), although gene flow was relatively high ($N_m = 7.1$).

Analysis of the same set of isolates with the VNTR markers showed that 60.8% of the VNTR loci were polymorphic in the 15ADON population and 49.3% were polymorphic in the 3ADON population. The gene diversity was 0.161 and 0.148 for the 15ADON and 3ADON populations, respectively. The LD value was 0.001 ($P = 0.30$) for the 15ADON population and 0.075 ($P = 0.001$) for the 3ADON population. AMOVA showed that genetic variation was 20% between populations and 80% among individuals within population. The Nei's unbiased genetic dis-

tance (D) was low (0.055) but the gene flow was also relatively low ($N_m = 2.04$). Significant genetic differentiation ($F_{st} = 0.197$, $P = 0.001$) was identified between the two populations.

DISCUSSION

Our study showed that the frequency of *F. graminearum* isolates with a 3ADON chemotype was very low (3%) in the old collection but increased by 15-fold in the new collection (Fig. 1A and B). This result is consistent with those reported previously (13,19,52), which demonstrated that an *F. graminearum* population with a 3ADON chemotype has emerged and increased since 1998. Gale et al. (13) had reported genetically divergent populations of *F. graminearum* in North Dakota and Minnesota, where 30 3ADON isolates (9.4%) were identified among 587 isolates collected in 1999 to 2000. Ward et al. (52) analyzed populations of *F. graminearum* in Canada and found that the 3ADON chemotype frequency in western Canada increased more than 14-fold between 1998 and 2004. They also demonstrated a dramatic longitudinal cline in which 3ADON isolates were significantly more common in eastern Canada than in western provinces of Canada. In addition, Guo et al. (19) analyzed *F. graminearum* samples collected from 15 locations of Manitoba in 2004 to 2005 and found that percentages of isolates with a 3ADON chemotype was 0 to 95.7%, depending on the locations sampled. These findings contradict earlier reports by Zeller et al. (55,56), who suggested that the *F. graminearum* population in North America is homogeneous and can be represented by local inocula in breeding programs. Lack of significant differentiation or subdivision among the *F. graminearum* populations analyzed by Zeller et al. (55,56)

Fig. 3. Deoxynivalenol (DON) accumulation in harvested grains of the three wheat genotypes (Grandin, Steele-ND, and ND 2710) averaged based on chemotype population (3-acetyl-deoxynivalenol [3ADON] or 15-acetyl-deoxynivalenol [15ADON]). The 3ADON population accumulated significantly more DON on Grandin ($P = 0.0006$) and ND2710 ($P = 0.0016$) than the 15ADON population. However, no significant difference ($P = 0.1057$) in DON accumulation was detected on Steele-ND between the two populations.

TABLE 3. Comparison of the 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON) populations of *Fusarium graminearum* in trichothecene production in rice culture and grains harvested from the inoculated wheat genotypes

Population ^y	Trichothecene (ppm) ^z					
	In rice culture			In grains		
	DON	3ADON	15ADON	DON	3ADON	15ADON
3ADON	241.1 ± 32.2	155.3 ± 24.8	1.3 ± 0.3	17.3 ± 2	0.7	–
15ADON	168.0 ± 39.7	1.8 ± 0.2	8.1 ± 1.8	7.6 ± 1.3	–	0.3

^y The 3ADON and 15ADON populations consisted of 13 and 12 isolates of *Fusarium graminearum*, respectively.

^z Mean of deoxynivalenol (DON) or its derivatives was calculated among the isolates used in each population; – = not detected.

might be due to absence or very low frequency of the 3ADON isolates in their samples collected from 1999 and 2000. The dramatic population changes in *F. graminearum* and significant increase of the 3ADON isolates might have not occurred in the United States until 2000.

Our population genetic analyses with AFLP and VNTR markers showed that the 15ADON isolates had higher gene diversity and more polymorphic loci compared with the 3ADON isolates. Further, a significant genetic differentiation was detected between the two populations and gene flow (N_m) between them was low based on VNTR markers. These results suggest that the 3ADON population is different from the prevalent 15ADON population and might have emerged more recently. However, the number of isolates used in the study was low. Analysis of more isolates from more counties of North Dakota is needed to reveal the relationship between the 3ADON isolates and 15ADON isolates in this region.

The origin of 3ADON isolates in the North American population is not known but it was suggested that they were introduced from other continents through contaminated seed (13,52). Gale et al. (13) compared 30 isolates of the 3ADON chemotype collected from North Dakota and Minnesota with a small sample of isolates collected from Italy and found significant genetic similarity between the two populations; therefore, they suggested that the 3ADON population in the Upper Midwest of the United States might have originated from Europe. Ward et al. (52) further

indicated that Canadian 3ADON populations were more closely related to Italian populations than to the sympatric 15ADON populations and, thus, concluded that the 3ADON populations in North America were due to a transcontinental introduction. However, if the 3ADON populations did originate from other continents, the first introduction might not have occurred recently because three toxic and relatively aggressive isolates with a 3ADON chemotype were identified among the isolates collected between 1979 and 1981 from Ontario, Canada (14,36), and two of these isolates were genetically connected to the 3ADON population from Prince Edward Island (52). We also identified three 3ADON isolates (R458, R459, and R1391) in the old collection during the 1980s from North Dakota, suggesting that 3ADON isolates were present in the Upper Midwest of the United States at least at the same time as they were in eastern Canada. It is not known where these old 3ADON isolates came from. Further studies are required to reveal their relationship to the new 3ADON isolates in ND and those previously reported in other regions of North America (13,52).

Our study indicated that 3ADON isolates accumulated significantly higher DON than 15ADON isolates in rice culture. This is consistent with the study of Ward et al. (52), which demonstrated that mean trichothecene accumulation was significantly greater for isolates with a 3ADON chemotype than observed for the 15W population with a 15ADON chemotype in vitro. We inoculated three wheat genotypes with a subset of isolates from each of the

Fig. 4. Correlation between disease severity (DS) and trichothecene production in rice culture and grains harvested from inoculated heads of the three wheat genotypes (Grandin, Steele-ND, and ND 2710). **A**, DS with total deoxynivalenol (DON) in rice culture; **B**, DS with 3-acetyl-deoxynivalenol (3ADON) in rice culture; **C**, DS with 15-acetyl-deoxynivalenol (15ADON) in rice culture; and **D**, DS with DON in grains.

two populations with a 3ADON or 15ADON chemotype and showed that the average trichothecene accumulation by 3ADON isolates inoculated on the susceptible wheat cv. Grandin was greater than that observed for the 15ADON isolates. Mean trichothecene accumulation on the moderately resistant wheat line ND 2710 also was significantly greater for 3ADON isolates than for 15ADON isolates. However, no significant difference in trichothecene accumulation on Steele-ND was observed between the two chemotypes. These results suggest that the two chemotypes may interact differently, with wheat genotypes having different levels or sources of FHB resistance. Similar results were observed in the study of Ward et al. (52), which showed that the mean DON accumulation was significantly higher for the 3ADON population than for the 15ADON population on the susceptible cv. Robin; however, this difference was not statistically significant on the moderately resistant cv. 5062 HR.

Our study also showed that the 3ADON population caused a significantly higher level of disease than the 15ADON population on Grandin and ND 2710, although no significant difference in disease level was observed between the two chemotypes when inoculated on Steele-ND. This is in contrast to the result of Ward et al. (52), who found no significant difference in pathogenicity between the two chemotypes on either of the two cultivars (Robin and 5062 HR) evaluated. The discrepancy between the results of Ward et al. (52) and ours was probably due to the inoculation methods used. Ward et al. (52) used spray inoculation whereas we used single-floret inoculation. Previous studies demonstrated that DON is not required for initial infection by *F. graminearum* to cause FHB but it facilitates the disease spread from an infected floret into neighboring florets of a spike (20,40). Therefore, the spray-inoculation method might not be able to identify the differences in aggressiveness between isolates with different levels of DON production. In a field experiment, we also did not observe significant differences in DS on Trooper (susceptible to FHB) or Alsen (moderately resistant to FHB) when spray inoculated with the 3ADON and 15ADON isolates, although the DON levels were significantly higher for the 3ADON isolates than for the 15ADON isolates (Ali et al., unpublished data).

The spring wheat genotype ND 2710 with FHB resistance derived from Sumai3 (11) showed a significantly higher DS when inoculated with the 3ADON population than with the 15ADON population but no significant DS difference was observed in Steele-ND (Fig. 2), a cultivar with FHB resistance from *T. dicoccoides* (26), when inoculated with the two chemotype groups. Similarly, the 3ADON isolates accumulated higher DON in grains of ND2710 and Steele-ND compared with the 15ADON isolates but a significant difference in DON accumulation between the two chemotype populations was only observed on ND 2710 (Fig. 3). These results strongly suggest that differences in aggressiveness and DON production between the chemotype groups vary with host genotype used. In the Northern Great Plains, most recently released wheat cultivars such as Alsen, Faller, and Glenn have FHB resistance derived from Sumai3 (11) whereas Steele-ND has resistance derived from *Triticum dicoccoides* (27). It remains to be investigated whether these wheat cultivars have different reactions to the two chemotypes and whether FHB resistance plays any role in the population shift of *F. graminearum*.

Significantly high correlations between DS and total DON production were observed in the three wheat genotypes inoculated with either of the two chemotypes. This is consistent with the results of previous studies (7,18,50). Mesterhazy et al. (29), Bai et al. (4), Paul et al. (38), and Ma et al. (22) showed a strong positive correlation between Fusarium-diseased kernels and total DON accumulation. The amount of total DON accumulation depends on the level of host resistance and is generally higher in susceptible cultivars (28,29). However, other studies had shown a zero or negative correlation between FHB severity and amount of DON accumulation in grains (3,9,23). The contradictions among those

studies could be due to the use of different hosts, isolates, or inoculation methods.

In summary, our results indicate that *F. graminearum* isolates with a 3ADON chemotype have increased very rapidly in recent years in North Dakota. They are more aggressive by causing more disease in wheat cultivars and produce a higher amount of total DON in grains harvested from inoculated spikes. This information has implications for the development of FHB-resistant wheat cultivars and disease management. First, the *F. graminearum* population in North America consists of chemotypes with different aggressiveness and mycotoxin productivity. It is postulated that changes in agricultural practices such as use of host resistance and fungicides may drive the pathogen populations to shift to those with greater aggressiveness and DON production. Thus, sampling and monitoring of *F. graminearum* populations periodically on local and national scales are still necessary. In addition, screening of resistance for FHB should be made with inocula combining representative isolates with different trichothecene types in breeding programs. This will ensure the development of cereal crops with broad resistance to FHB pathogens. Finally, wheat genotypes with different sources of FHB resistance may react differentially to different chemotypes in DS and DON accumulation. This highlights the need and importance of using different sources of host resistance in combating the disease.

ACKNOWLEDGMENTS

This research work was funded partially by the Agricultural Experiment Station of North Dakota State University and the U.S. Wheat and Barley Scab Initiative. We thank R. W. Stack (retired) for providing the *Fusarium graminearum* isolates collected before 2000; K. Benson for mycotoxin analysis of the samples; J. Mulins, J. Hansen, S. Ali, and Y. Leng for assistance in the greenhouse inoculation; and J. Faris and M. McMullen for review of the manuscript before submission.

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