Fusarium Damage in Cereal Grains from Western Canada. 1. Phylogenetic Analysis of Moniliformin-Producing *Fusarium* Species and Their Natural Occurrence in Mycotoxin-Contaminated Wheat, Oats, and Rye

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Ⓢ *Supporting Information*

ABSTRACT: Harvest samples of common wheat (*Triticum aestivum*), oats (*Avena sativa*), and rye (*Secale cereale*) from producers in western Canada were analyzed for fungal infection by toxigenic *Fusarium* species and contamination by trichothecenes and moniliformin (MON). *Fusarium graminearum* and *F. avenaceum* were the two most frequently isolated species from samples of rye and wheat collected in 2010. *F. poae* and *F. sporotrichioides* were more commonly detected in randomly selected oat seeds. Other toxigenic *Fusarium* species including *F. acuminatum*, *F. culmorum*, and *F. pseudograminearum* as well as *Phaeosphaeria nodorum* (a.k.a. *Septoria nodorum*) were recovered primarily from fusarium-damaged kernels of wheat. Pure cultures of *F. avenaceum*, *F. acuminatum*, and other related species known to produce moniliformin were isolated from incubated seeds based on micro- and macromorphological criteria. The phylogenetic analysis inferred from partial DNA sequences of the *acl1* and *tef-1α* genes revealed two major clades representing *F. avenaceum* and *F. acuminatum*, respectively. These clades comprised all Canadian isolates of the two species and a number of reference cultures studied earlier for their propensity to form moniliformin *in vitro* and *in planta*. However, some reference cultures previously reported to produce significant amounts of moniliformin formed minor phylogenetic lineages that represent rather distinct but closely related species. Concomitantly, cereal samples were analyzed for the presence of deoxynivalenol and moniliformin. These two *Fusarium* toxins were observed most frequently in common wheat, at concentrations up to 1.1 and 4.0 mg/kg, respectively. There was no apparent relationship between moniliformin concentrations and detection of *F. avenaceum* and *F. acuminatum* in rye and oat samples. Geographical analysis of the distribution of moniliformin and *F. avenaceum* and *F. acuminatum* across the Canadian Prairies also did not indicate a strong relationship.

KEYWORDS: *Fusarium avenaceum, F. arthrosporioides, F. acuminatum, F. flocciferum, F. reticulatum, trichothecenes, deoxynivalenol*

■ INTRODUCTION

In the Western Canadian Prairie provinces of Alberta, Saskatchewan, and Manitoba, three species of *Fusarium* are associated with fusarium head blight (FHB): *F. graminearum*, *F. culmorum*, and *F. avenaceum*.[1−3] These species are known to cause disease symptoms such as fusarium-damaged kernels (FDK) on a number of cereal grains including wheat and barley. *F. graminearum* and *F. culmorum* also can produce mycotoxins such as the well-studied deoxynivalenol (DON); *F. avenaceum* has the ability to produce the less well known moniliformin (MON).[4] The diversity of *Fusarium* species on Canadian grains, however, is much more comprehensive and includes other members of the genus such as *F. acuminatum*, *F. equiseti*, *F. poae*, *F. sporotrichioides*, and *F. verticillioides*.[5,6] Most of these species are also potent producers of other less studied mycotoxins, including beauvericins, enniatins (ENNs), nivalenol, and HT-2 and T-2 toxins.[7]

Moniliformin, as one of the emerging *Fusarium* toxins in grains, recently has spurred the interest of researchers, regulatory agencies, and industry worldwide. For example, the European Food Safety Authority recently closed a call for data regarding the occurrence of MON in food and feed

[http://www.efsa.europa.eu/en/dataclosed/call/datex101020b.htm]. Moniliformin was first discovered in 1973 during studies on southern corn leaf blight.[8] In 1970, the disease caused great harvest losses in the southeastern and the midwestern United States,[9] and the name "moniliformin" was derived from the causal agent of the disease, *Fusarium moniliforme* (later correctly identified as *F. proliferatum*). Various other *Fusarium* species, mainly in the section *Liseola* and the *F. acuminatum*/ *F. avenaceum*/*F. tricinctum* phylogenetic species complex have been shown to produce MON *in vitro* and *in vivo*.[4,10,11] The first study on the propensity of MON formation by Canadian isolates of *Fusarium* confirmed *F. subglutinans* and *F. verticillioides* as producers in corn.[12] Later, Abramson et al.[13] and Clear et al.[14] showed that almost all Canadian isolates of *F. avenaceum* studied were toxigenic in rice culture, producing MON at ppm levels. European isolates of *F. avenaceum* seemed more variable in their propensity to form MON *in vitro*.[15] Less than half of the strains

tested in culture on corn meal agar produced MON at ppm levels. However, a fungal strain very closely resembling *F. avenaceum* morphologically but originating from Asia produced levels of MON approximately 20 times greater *in vitro* and in the plant. At these levels, the isolate fell in the range of some of the most potent producers of MON belonging to the species *F. proliferatum* and *F. subglutinans*.[15] The test results by Schütt[15] did not reveal a common pattern of MON production for the morphological species studied, indicating that the chemotype was a highly variable trait of the phenotype.

The natural occurrence of MON has been reported from a number of countries in Africa, Asia, Europe, and North America.[16−18] In Western Canadian wheat, MON was first reported to occur naturally by Clear et al.[19] For 2002, the authors reported only one composite of harvest survey samples of Canada Western Red Spring wheat from Manitoba containing MON (0.07 mg/kg). In contrast, about half of the composite samples of Canada Western Amber Durum (CWAD) wheat from crop districts in Alberta, Manitoba, and Saskatchewan contained MON at levels between 0.04 and 0.13 mg/kg in that study.[19]

The focus of this study was the natural occurrence and population structure of *Fusarium* species, including *F. acuminatum*, *F. arthrosporioides*, *F. avenaceum*, and closely related taxa known to produce MON.[4] In this study, we further characterized some of the *Fusarium* strains tested for MON production by Schütt[15] using DNA sequences of two single-copy nuclear genes. By elucidating the phylogenetic structure within the *F. acuminatum*/*F. avenaceum*/*F. tricinctum* species complex on the basis of genealogical concordance, we wanted to trace the evolutionary history of MON production in the clade. Furthermore, in comparison with reference material from the *Fusarium* culture collection at the Julius-Kühn-Institute (formerly BBA, Berlin, Germany) studied by Lamprecht et al.[20] and Schütt[15] for their propensity to form MON, *Fusarium* isolates from western Canada were assessed for their phylogenetic relationship to known MON producers. This indirect approach was taken, as none of the biosynthetic and regulatory genes involved in the polyketide pathway of MON formation have been identified yet.[21]

Concomitantly, we tested samples of small-grain cereals, in particular oats, rye, and common wheat, collected at harvest in 2010 from various districts across the Canadian Prairies for the presence of MON, DON, and other *Fusarium*-produced mycotoxins. Heavy rainfalls across most of the Canadian Prairies during the growing season of 2010 (regionally >200% above long-term average) and the presence of excessive moisture in the fields have allowed us to draw from a good number of cereal samples severely damaged by *Fusarium*.[22]

■ MATERIALS AND METHODS

Grain Samples. Samples of oats ($n = 62$), rye ($n = 13$), and wheat ($n = 26$) from the 2010 harvest on the Canadian Prairies were obtained from the Canadian Grain Commission (CGC) annual Harvest Sample Program as well as producer groups for mycotoxin and fungal analysis. The wheat samples represented four different classes: Canada Western Red Spring (CWRS; $n = 13$), Canada Western Soft White Spring (CWSWS; $n = 6$), Canada Western Hard White Spring (CWHWS; $n = 6$), and Canada Prairie Spring Red (CPSR; $n = 1$).

Harvest Sample Program samples consisted of grain that was voluntarily sampled at harvest by producers in the provinces of Manitoba, Saskatchewan, and Alberta and submitted to the CGC. Sampling instructions were provided to producers prior to harvest and indicated that it is preferable to sample flowing grain during unloading of trucks by passing an open container completely across the grain stream every

30−60 s. An alternate method of probing a truckload in at least two locations was also given. Producers are then directed to combine collected increments in a bucket and mix well and to provide approximately 500 g of composite sample to the CGC.

Once received, samples had dockage removed via a Carter Day cleaner, tested for moisture and protein content, examined by CGC inspectors, and given an unofficial grade. They were then stored at room temperature in paper envelopes until chemical and biological analyses were performed. Before sample preparation, each sample was passed through a centrifugal seed divider (Pascall Engineering Co. Ltd., England, UK) several times to ensure a good mixing and then split in halves. One half was retained for fungal analysis, and the other half was used for chemical analysis.

Visible *Fusarium* Damage in Wheat. For wheat, fusarium-damaged kernels were estimated and weighed by CGC grain inspectors according to grading specifications published in Canada's official grain grading guide (http://www.grainscanada.gc.ca/oggg-gocg/04/oggg-gocg-4-eng.htm). Seeds of common wheat moderately affected by FHB were identified visually based on mycelial growth around the germ and in the broad crease. Occasionally, other symptoms such as a wrinkled seed coat, a broad crease, or a chalky white appearance were noticed. FDK with severe symptoms showed abundant mycelial growth visible on both seed surfaces, with some pink discoloration at the germ. The seed had a shriveled, chalky white appearance and a broadened crease (http://www.grainscanada.gc.ca/guides-guides/identification/fusarium/fw-fb-eng.htm).[23]

Fungal Analysis and Reference Strains of *Fusarium*. Fusarium-damaged kernels were removed from each wheat sample and prepared for plating onto potato dextrose agar (PDA; Difco). Because fewer visible symptoms of FHB are evident in oat and rye, 100 seeds were selected at random for plating. Prior to plating, the seeds were soaked in a 0.3% sodium hypochlorite solution for 1 min and then dried in a laminar-flow hood. Ten seeds per 9 cm plastic Petri dish were placed onto PDA and subsequently incubated for 5 days at room temperature under a 12 h on/off cycle of near-ultraviolet and fluorescent light or darkness. Fungal identifications were done according to the methods described previously.[24] Fusaria developing from the seed were each transferred to a new 6 cm plastic Petri dish and grown on PDA for 7 days at room temperature. Numerous pure cultures of *F. avenaceum* and *F. acuminatum* were isolated from incubated seeds based on macro- and micromorphological criteria (see supplementary table for sample information). Morphological observations of colonies and microscopic characters were based on strains grown on PDA and synthetic low-nutrient agar (SNA)[25] in the laboratory at room temperature under a 12 h on/off cycle of near-ultraviolet and fluorescent light or darkness. A total of 75 *Fusarium* isolates from western Canada were included in the phylogenetic study. In addition, 30 *Fusarium* strains from the fungal culture collection at the Julius-Kühn-Institute (formerly BBA) in Braunschweig, Germany, were incorporated as reference material from nine different countries in Africa, Asia, and Europe. Of these BBA strains, 20 *Fusarium* cultures had been tested for moniliformin production previously by Lamprecht et al.[20] and Schütt.[15] The author used an agar-plug method and direct chemical analysis to determine the propensity to form MON *in vitro*.

DNA Extraction, PCR, and DNA Sequencing. Mycelia of pure cultures were scraped from colonies using a sterile scalpel and transferred to 96-well extraction plates. DNA extraction and purification were performed using NucleoSpin 96 Plant II kit (Macherey-Nagel) according to the manufacturer's instructions with the addition of a single 4.5 mm steel airgun shot (Marksman) bead to each well to better facilitate disruption of the fungal cells using a mixer mill (Retsch, Germany). DNA concentration and quality were determined by a Nanodrop ND-1000 spectrometer (Thermo Scientific, Wilmington, DE, USA). Aliquots were diluted to 1−5 ng/μL of DNA template just before preparation of PCR reactions.

The partial nuclear translation elongation factor 1 alpha (*tef-1α*) and the larger subunit of the ATP citrate lyase (*acl1*) were amplified following the protocol of de Cock and Lévesque[26] using the primer combinations EF1/EF2[27] and acl1-230up/acl1-1220low,[28] respectively, in a total reaction volume of 20 μL. PCR reactions were placed in a

Biometra TProfessional TRIO thermocycler (Goettingen, Germany) and processed with the published temperature profiles. For forward and reverse strands, sequencing reactions were performed directly without cleaning PCR amplicons, using a BigDye sequencing kit on an ABI3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Contig sequences were assembled using Sequencher 5.0 (Gene Codes, Ann Arbor, MI, USA) and aligned manually using BioEdit 7.[29] Introns of *acl1* and *tef-1α* gene sequences were included in the final alignment consisting of 1563 bp and 105 taxa. DNA sequences of 74 *Fusarium* isolates were generated in this study and deposited in GenBank (accession numbers KC999478–KC999615). For 31 additional strains, partial *acl1* and *tef-1α* gene sequences were taken from Niessen et al.[44]

Phylogenetic Analysis. The combined data set of the *acl1* and *tef-1α* gene sequences was used to search for the best maximum likelihood (ML) tree employing the MEGA 5.1 software.[30] The GTR + I + G nucleotide substitution model was selected, which assumes an estimated proportion of invariant sites and eight gamma-distributed rate categories to account for rate heterogeneity across sites. ML heuristic phylogenetic analysis was performed with a starting tree generated by maximum parsimony (MP) via 10 stepwise, random addition sequences using all sites and the near-neighbor interchange (NNI) method.

Nonparametric bootstrapping of 500 ML pseudoreplicates of the data was used to assess clade support under the aforementioned parameters employing MEGA 5.1. ML bootstrap probabilities (ML-BP) for the splits were mapped onto the best ML phylogenetic tree inferred under the GTR + I + G nucleotide substitution model. MP bootstrap probabilities (MP-BP) were assessed by 1000 heuristic pseudoreplicates using tree−bisection−reconnection with the same settings as above employing MEGA 5.1.

Mycotoxin Analysis. Samples were analyzed for a number of *Fusarium*-related mycotoxins: MON, DON, 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV), HT-2 toxin, T-2 toxin, fusarenon-x, and diacetxoyscirpenol.

The entire mass of grain available for chemical analysis (between 200 and 300 g) was ground using a commercial coffee grinder (model KR 804, Ditting Maschinen AG, Switzerland). The sample was ground fine enough that ≥85% of mass passed through a US 50 sieve (nominal sieve openings of 300 μm). Two 10 g portions of ground grain were taken for mycotoxin analysis. One portion was used for MON analysis, the other 10 g portion went to the analysis of the other mycotoxins.

MON was analyzed in cereal samples using high-performance liquid chromatography with photodiode array detection (HPLC-PDA) based on a method described by Uhlig et al.[31] Briefly, the 10 g portion of ground sample was extracted with 100 mL of 84:16 (v/v) acetonitrile/water on a shaker bed for 30 min. The extract was then evaporated to dryness on a rotary evaporator and reconstituted in methanol. The extract was cleaned using strong anion exchange SPE (Bond Elut-SAX, Agilent Technologies). MON was eluted with 3.5% tetrabutylammo-mium hydrogen sulfate (w/v) in 0.2 M aqueous KH_2PO_4 adjusted to pH 7.0 using 5 M aqueous KOH. The eluate was made up to 3.0 mL using purified H_2O and then filtered through a 0.45 μm × 13 mm PVDF syringe filter.

The sample was chromatographed on a Waters 2695 HPLC system connected to a Waters 996 PDA. The system was operated using a Millenium 32 chromatography data system. The HPLC was fitted with a 3.9 × 150 mm Nova-Pak C_{18} HPLC cartridge column (Waters) connected to a Nova-Pak C_{18} Sentry Guard-Pak column. The analytical column was held at a temperature of 30 °C, and the sample tray at 20 °C. Chromatography was performed using isocratic conditions and a mobile phase of 92:8:1 (v/v/v) purified H_2O, acetonitrile, and ion pairing reagent. The ion pairing reagent consisted of 7% tetrabutyl-ammomium hydrogen sulfate (w/v) in 0.4 M aqueous KH_2PO_4 adjusted to pH 7.0 using 5 M aqueous KOH. The PDA detector scanned from 200 to 350 nm at a rate of 1.0 spec/s at a resolution of 2.4 nm.

MON was considered to be detected if the retention time of the peak was within 0.1 min of the average retention time in the calibration standards, the peak signal-to-noise ratio was greater than 9, and acceptable peaks were observed at the quantitation wavelength of 229 nm, as well as the confirmation wavelengths of 249 and 260 nm.

A six-point calibration curve of MON peak area versus mass of MON injected with an $R^2 \geq 0.99$ was constructed for quantitation using standards prepared with the sodium-MON salt (Sigma Aldrich) in dilute ion pairing reagent. Concentrations in samples were calculated as the MON anion and not the sodium salt.

A number of quality control measures were used to ensure quality MON data were generated. For batch runs that contained more than 10 samples, one additional calibration standard was inserted with the samples after each 10 samples injected. The concentration of MON was calculated using the calibration curve, and a tolerance of ±20% of the nominal value was set. In addition, a grain sample previously analyzed and known to be free of MON was run with each batch as a blank. Finally, each batch contained a blank sample fortified with MON at a concentration of 0.20 μg/kg and an in-house reference material naturally containing approximately 0.3 μg/kg MON. Prior to extraction the fortified sample sat in a fumehood for 30 min and was then subjected to the same treatment as the other samples in the batch. Recoveries of MON were calculated from the concentration of MON quantitated in the fortified blank.

Analysis of DON, 3-ADON, 15-ADON, NIV, HT-2 toxin, T-2 toxin, fusarenon-x, and diacetxoyscirpenol was based on the method of Tacke and Casper[32] and was performed as described in Clear et al.[19] Ground CWAD was extracted with a solution of acetonitrile/water. An aliquot of the extract was cleaned using C_{18} reverse phase bonded silica gel and aluminum oxide solid phase extraction. An aliquot of the solid phase extraction eluate was evaporated to dryness, and analytes were derivatized into their trimethylsilyl derivatives. Telodrin was added as an internal standard to the derivatized extract for use as an internal standard in the GC-MS analysis. The prepared samples were chromato-graphed on an Agilent capillary 6890 GC equipped with a Hewlett-Packard 5973 mass selective detector and a Hewlett-Packard 7673A autosampler. The GC contained a DB35-MS 30 m × 0.25 mm × 0.25 μm column (Agilent). Sample was injected in pulsed splitless mode using a pulse pressure of 40.0 psi.

Four ions were monitored for each analyte. Analytes were con-sidered to be positively identified and quantified if their retention times were within 0.1 min of the average retention time of the cor-responding analyte in the external calibration standards; the peak had a signal-to-noise ratio greater than 9:1; and the ratio of qualification to quantitation ions was within acceptable tolerances.[10,33] The analyte responses were normalized to the telodrin responses during calculation of concentrations. Finally, the analyte concentrations were recovery corrected using the percent recovery of analyte from a fortified wheat blank run with every sample batch.

Similar quality control measures were used to monitor the analyses of the other *Fusarium*-produced mycotoxins as for MON. A certified reference material for DON in wheat (Trilogy Analytical Laboratory, Washington, MO, USA) was also analyzed in each batch to monitor the performance of the DON analyses.

■ RESULTS

Frequency of FDK in Wheat. In 2010, all common wheat classes grown in western Canada were affected by *Fusarium*. In total, 17.2% of the 3803 CWRS samples submitted and inspected as part of the Harvest Sample Program contained FDK. *Fusarium* damage was even more severe in Canada Western Red Winter (CWRW) wheat with an incidence of 23.2% in 99 samples inspected (data not shown). In comparison to Harvest Sample Program results from 2003 to 2012, in 2010 a larger proportion of CWRS samples from Alberta was affected by *Fusarium*, and the degree of damage was more severe in central Saskatchewan and many parts of Alberta (Table 1). For example, in crop districts 1, 2, 4, and 5 in Alberta and 3BN, 4B, 6, 7, and 8 in Saskatchewan, average severity of fusarium damage ranged from 1.50% to 2.66% in up to 29.3% of all CWRS samples. Here, we define average severity as percentage (by weight) of FDK in samples affected by *Fusarium*. The percentage of samples containing FDK out of all samples inspected reflects

Table 1. Frequency of Detection of Fusarium-Damaged Kernels and Severity of Fusarium Damage Observed in Canada Western Red Spring (CWRS) Wheat Grown in Manitoba, Saskatchewan, and Alberta Crop Districts and Submitted to the Canadian Grain Commission Harvest Sample Program from 2003 through 2012[a]

crop district	2012	2011	2010	2009	2008	2007	2006	2005	2004	2003
					Manitoba (MB)					
1	67.4	47.8	46.5	12.1	26.5	15.0	6.9	69.0	23.6	18.6
	1.03	2.01	1.21	0.45	0.67	0.80	0.62	1.70	1.28	0.08
	(95)	(46)	(114)	(140)	(117)	(113)	(145)	(116)	(110)	(172)
2	57.1	16.7	39.7	22.7	49.3	11.8	0.8	58.8	18.3	7.7
	1.05	0.77	1.10	0.51	0.85	1.19	1.00	1.20	1.50	0.07
	(91)	(42)	(73)	(66)	(67)	(93)	(123)	(119)	(93)	(143)
3	61.0	15.0	44.7	23.2	53.5	16.0	2.3	58.3	14.0	16.2
	0.79	0.21	1.14	0.60	1.75	1.24	0.27	1.55	2.06	0.08
	(82)	(60)	(85)	(82)	(71)	(75)	(129)	(120)	(86)	(136)
4	54.4	7.4	26.4	6.7	70.8	6.0	3.3	35.9	4.3	6.6
	0.44	0.88	0.91	0.81	1.69	1.33	0.40	0.70	0.70	0.17
	(57)	(54)	(53)	(60)	(48)	(50)	(61)	(64)	(23)	(61)
5	41.5	14.5	42.0	3.8	32.6	21.6	0.0	9.5	15.8	14.5
	0.77	0.91	1.49	0.42	0.99	0.49	0.00	1.02	0.92	0.09
	(94)	(110)	(69)	(78)	(89)	(74)	(76)	(74)	(38)	(69)
6	51.9	0.0	30.5	32.5	50.6	17.1	0.0	10.7	5.2	30.9
	0.81	0.00	0.93	1.60	2.13	0.67	0.00	0.51	2.80	0.27
	(81)	(59)	(59)	(77)	(77)	(76)	(114)	(131)	(58)	(97)
7	29.7	13.0	62.2	49.6	74.6	14.1	11.6	55.9	17.9	63.6
	0.69	0.36	1.39	1.51	1.78	0.79	0.72	1.54	1.14	0.36
	(145)	(123)	(119)	(119)	(114)	(92)	(121)	(93)	(78)	(173)
8	29.4	21.7	62.3	51.5	56.9	18.7	4.7	66.3	18.9	68.2
	0.66	0.70	0.89	1.47	1.48	0.75	0.40	1.61	2.14	0.35
	(143)	(138)	(151)	(132)	(123)	(123)	(149)	(160)	(106)	(239)
9	17.9	2.0	87.5	90.9	80.5	11.4	4.0	44.4	15.6	76.5
	0.41	0.20	1.81	3.49	1.72	0.95	2.14	0.90	1.25	0.45
	(56)	(49)	(48)	(33)	(41)	(44)	(50)	(45)	(32)	(85)
11	10.0	0.0	57.1	94.7	75.0	14.3	9.5	52.9	16.7	70.3
	0.22	0.00	1.62	1.76	6.65	0.92	0.39	0.93	2.20	0.42
	(20)	(11)	(21)	(19)	(20)	(21)	(42)	(34)	(30)	(64)
12	13.3	30.0	50.0	38.5	18.8	7.1	5.9	16.7	6.7	25.7
	0.25	1.00	1.39	2.51	2.27	0.50	1.20	1.15	0.08	0.40
	(15)	(20)	(10)	(13)	(16)	(14)	(17)	(12)	(15)	(35)
					Saskatchewan (SK)					
1A	52.8	10.3	14.5	1.6	3.5	2.4	0.0	12.3	9.4	6.8
	0.82	0.81	1.20	0.45	0.13	0.55	0.00	0.68	0.41	0.25
	(72)	(29)	(83)	(62)	(57)	(82)	(91)	(130)	(85)	(118)
1B	54.7	4.8	12.8	0.0	20.5	8.6	0.0	11.3	1.9	3.4
	0.85	0.15	1.10	0.00	1.04	1.09	0.00	0.65	0.00	0.04
	(53)	(21)	(47)	(49)	(44)	(81)	(95)	(97)	(54)	(146)
2A	46.2	0.0	0.0	6.7	0.0	0.0	1.4	2.8	0.0	4.1
	0.59	0.00	0.00	0.38	0.00	0.00	0.35	0.38	0.00	0.04
	(39)	(17)	(40)	(30)	(28)	(30)	(74)	(71)	(43)	(98)
2B	56.6	4.4	11.1	3.6	6.4	0.0	0.0	3.2	1.9	0.6
	1.24	1.03	1.37	0.50	0.64	0.00	0.00	1.39	0.80	0.12
	(83)	(90)	(90)	(83)	(94)	(112)	(154)	(125)	(106)	(168)
3AN	3.3	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.9
	0.23	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06
	(30)	(33)	(24)	(17)	(22)	(34)	(29)	(36)	(25)	(35)
3AS	1.7	5.4	3.0	0.0	0.0	0.0	0.0	1.9	0.9	0.0
	0.10	0.18	0.65	0.00	0.00	0.00	0.00	0.18	0.40	0.00
	(58)	(56)	(99)	(50)	(64)	(98)	(131)	(106)	(109)	(104)
3BN	1.7	1.3	1.7	0.0	0.0	0.0	0.0	2.9	2.4	0.0
	0.80	0.35	2.66	0.00	0.00	0.00	0.00	0.87	0.35	0.00
	(58)	(76)	(118)	(87)	(77)	(94)	(143)	(105)	(83)	(110)
3BS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.1	0.0
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00
	(33)	(38)	(38)	(19)	(38)	(41)	(66)	(47)	(32)	(50)

Table 1. continued

crop district	2012	2011	2010	2009	2008	2007	2006	2005	2004	2003
					Saskatchewan (SK)					
4A	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.3	0.0
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.65	0.00
	(25)	(25)	(36)	(20)	(19)	(28)	(29)	(25)	(15)	(20)
4B	0.0	3.7	3.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.00	0.61	1.85	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	(39)	(27)	(65)	(22)	(32)	(36)	(62)	(53)	(37)	(42)
5A	68.5	8.7	17.5	11.8	25.5	11.4	0.0	6.9	1.8	2.7
	0.99	0.58	1.24	0.56	1.40	0.48	0.00	0.54	0.25	0.04
	(127)	(126)	(143)	(144)	(153)	(167)	(189)	(188)	(111)	(261)
5B	58.5	26.5	28.9	14.8	23.0	2.0	0.7	4.3	3.0	3.2
	0.77	0.64	1.35	0.39	0.83	0.80	0.24	0.26	0.22	0.14
	(135)	(102)	(76)	(81)	(100)	(99)	(153)	(138)	(99)	(185)
6A	62.5	8.2	21.5	15.9	2.0	0.0	0.0	3.2	0.8	1.6
	1.06	0.30	1.74	0.46	0.60	0.00	0.00	0.82	0.20	0.04
	(112)	(122)	(107)	(69)	(98)	(112)	(169)	(155)	(130)	(247)
6B	62.9	13.2	13.9	8.5	2.1	1.7	0.0	0.0	0.0	1.6
	1.33	0.80	1.69	0.44	0.40	0.90	0.00	0.00	0.00	0.02
	(105)	(106)	(115)	(82)	(97)	(115)	(158)	(140)	(101)	(183)
7A	37.8	3.6	14.7	7.9	0.0	1.0	0.0	0.8	1.9	0.0
	1.01	0.32	2.02	0.69	/0.00	1.00	0.00	0.20	0.70	0.00
	(82)	(110)	(109)	(76)	(77)	(99)	(145)	(119)	(105)	(84)
7B	37.0	2.8	7.9	1.1	0.8	0.0	0.5	1.5	0.0	0.0
	0.64	0.20	1.51	0.32	0.60	0.00	0.40	0.63	0.00	0.00
	(81)	(109)	(114)	(93)	(122)	(130)	(190)	(136)	(107)	(160)
8A	75.0	34.0	29.3	16.5	13.4	6.0	4.1	3.0	0.0	2.9
	1.86	1.25	1.69	0.81	0.60	1.14	0.61	1.08	0.00	0.07
	(136)	(150)	(157)	(121)	(157)	(133)	(220)	(166)	(108)	(245)
8B	80.5	17.1	20.3	9.1	11.4	1.8	1.4	4.6	1.1	2.6
	1.43	0.65	1.50	0.51	0.50	0.33	0.80	0.86	0.15	0.02
	(113)	(111)	(118)	(77)	(88)	(109)	(143)	(130)	(94)	(192)
9A	57.0	6.8	12.5	4.6	2.4	0.7	1.4	5.7	0.0	1.0
	0.93	0.36	1.00	0.51	0.44	0.90	0.60	0.81(1	0.00	0.12
	(107)	(117)	(104)	(87)	(127)	(142)	(147)	23)	(85)	(209)
9B	24.4	0.0	11.3	0.0	0.0	0.0	0.0	6.7	0.0	0.0
	0.69	0.00	1.07	0.00	0.00	0.00	0.00	0.94	0.00	0.00
	(45)	(58)	(62)	(47)	(67)	(77)	(71)	(60)	(40)	(83)
					Alberta (AB)					
1	7.5	2.2	3.8	9.6	0.0	0.0	0.0	1.3	0.8	1.0
	0.55	0.18	1.50	1.67	0.00	0.00	0.00	0.98	1.20	0.02
	(106)	(93)	(133)	(73)	(105)	(112)	(140)	(157)	(123)	(206)
2	18.6	10.2	7.6	10.8	0.4	0.3	0.6	0.0	0.5	0.3
	0.70	1.06	1.50	1.10	0.95	0.40	0.78	0.00	0.80	0.06
	(279)	(265)	(224)	(222)	(226)	(343)	(309)	(259)	(188)	(342)
3	5.0	6.1	0.0	1.9	2.1	0.0	0.0	1.0	1.0	0.7
	0.37	0.83	0.00	0.62	0.88	0.00	0.00	0.90	0.50	0.02
	(60)	(82)	(89)	(106)	(94)	(137)	(127)	(105)	(100)	(143)
4	12.9	4.8	4.3	0.7	0.0	0.0	0.0	2.3	1.2	1.4
	0.64	0.64	1.65	0.47	0.00	0.00	0.00	1.34	0.37	0.05
	(326)	(357)	(468)	(409)	(504)	(514)	(480)	(388)	(254)	(357)
5	19.4	3.5	6.4	0.0	1.6	0.0	0.0	2.5	2.3	2.9
	0.65	0.73	2.51	0.00	0.70	0.00	0.00	0.70	0.30	0.15
	(62)	(85)	(94)	(66)	(61)	(64)	(68)	(80)	(44)	(69)
6	8.8	5.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.3
	0.86	0.48	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
	(34)	(34)	(32)	(27)	(28)	(22)	(33)	(33)	(18)	(19)
7	1.9	2.3	0.8	0.0	0.4	0.4	0.0	0.3	2.0	2.1
	0.12	1.09	1.35	0.00	0.50	0.50	0.00	0.00	1.18	0.14
	(369)	(302)	(256)	(229)	(266)	(251)	(376)	(333)	(249)	(242)

[a]From top to bottom in each cell, data are provided for mean percentage of samples containing fusarium-damaged kernels (incidence), mean % (mass basis) fusarium-damaged kernels in samples containing damaged kernels (severity), and number of samples inspected (in parentheses).

the incidence of fusarium damage. In previous years, fusarium damage incidence rarely exceeded 15%, and average severity was most often less than 1.50%, especially in CWRS samples from Alberta and Saskatchewan. In 2012, the incidence of fusarium damage increased to 80.5% in parts of Saskatchewan. However, in most areas across western Canada, average severity did not exceed 1.4%. In the past (e.g., 2008, 2009), fusarium damage incidence occasionally exceeded 80% with an average severity of up to 3.5%, especially in southern Manitoba. Since the late 1990s/early 2000s, fusarium damage often has been the top degrading factor for CWRS grown in southern Manitoba.

Identification of *Fusarium* Isolates. From 13 wheat samples, five *Fusarium* species were recovered from a total of 134 FDK with occasionally more than one species isolated from an individual seed (see supplementary table). The most commonly recovered *Fusarium* species was *F. avenaceum*, which was detected in 10 wheat samples (from a total of 38 FDK). Three of these samples also contained individual FDK that were caused by *F. acuminatum*. *Fusarium graminearum* was recovered from a total of seven wheat samples and was predominant (present in >50% of FDK) in four samples from Manitoba or eastern Saskatchewan. Similarly, *F. culmorum* was detected in four samples and was the predominant species in one CWSWS sample from southern Alberta. Another DON-producing species, *F. pseudograminearum*, was found once on a single FDK from a CWRS sample from central Saskatchewan. Other *Fusarium* species, including *F. equiseti*, *F. poae*, or *F. sporotrichioides*, were not recovered from wheat FDK. In central Alberta, however, *Phaeosphaeria nodorum* (a.k.a. *Septoria nodorum* or *Stagonospora nodorum*) was the most frequently detected fungus associated with fusarium-damaged wheat in 2010 (recovered from a total of 122 FDK).

From a total of 1200 rye seeds randomly selected for plating, only 90 were contaminated with *Fusarium*. *F. avenaceum* (48 seeds) and *F. graminearum* (34 seeds) were more frequently detected than any other species. *F. acuminatum*, *F. poae*, and *F. sporotrichioides* were recovered only at low frequencies (in <1% of plated seeds).

In the oat samples, 376 seeds were infected with one of nine *Fusarium* species recovered from the 2700 seeds randomly selected for plating. Approximately half of the infected seeds (179 seeds) were contaminated with *F. poae*. With a frequency of almost 7%, it was the predominant species of *Fusarium* on oat in the analyzed samples. *F. graminearum* (83 seeds), *F. avenaceum* (57 seeds), and *F. sporotrichioides* (31 seeds) were three additional species frequently detected on oat (see supplementary table). Other species recovered at low frequencies (in <1% of plated seeds) included *F. acuminatum*, *F. culmorum*, *F. equiseti*, *F. solani*, and *F. tricinctum*. The latter three species were found only in oats in this study. *Phaeosphaeria nodorum*, known to cause FDK-like symptoms in wheat, was detected in a small number of seeds (n = 14) and was therefore not commonly associated with oat or rye.

Phylogenetic Analysis of *Fusarium* Species. The best ML tree inferred from the partial DNA sequences of the *acl1* and *tef-1α* genes of fungal isolates morphologically identified as *F. acuminatum*, *F. arthrosporioides*, and *F. avenaceum* is shown in Figure 1. The estimated value of the shape parameter for the discrete gamma distribution was 0.2025. Mean evolutionary rates in the eight gamma categories were 0.0000, 0.0011, 0.0118, 0.0588, 0.2038, 0.5770, 1.5301, and 5.6173 substitutions per site. The nucleotide frequencies were A = 22.61%, T = 24.69%, C = 29.05%, and G = 23.65%. The maximum log likelihood for
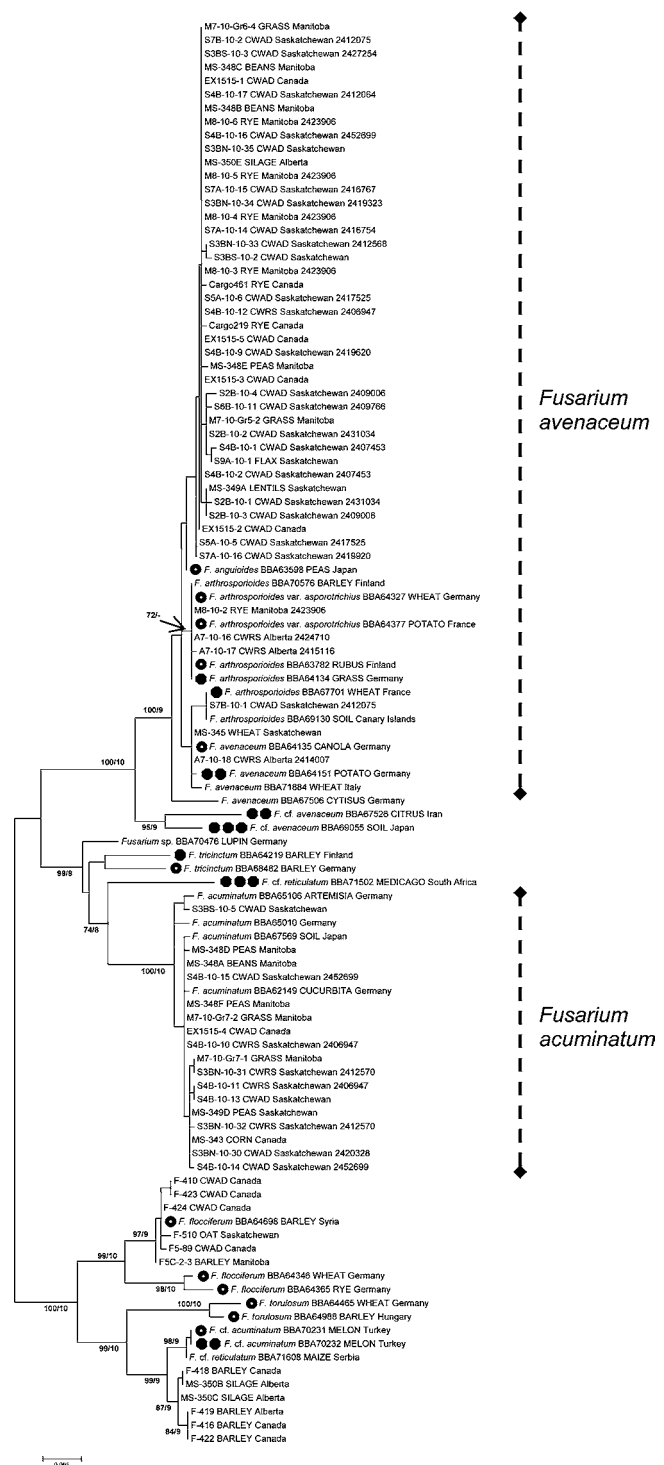

Figure 1. Phylogenetic relationship of *F. avenaceum*, *F. acuminatum*, *F. flocciferum*, and closely related species shown in a maximum likelihood (ML) tree under the GTR + I + G nucleotide substitution model inferred from combined *acl1* + *tef-1α* gene sequence data set. Negative-log likelihood (−lnL) of the ML tree is −4232.95. ML-BP/MP-BP values of >70% are given near internodes. Reference strains tested for MON production *in vitro* by Lamprecht et al.[20] and Schütt[15] are indicated by symbols (○, no MON production; ●, < 100 mg/kg, ●●, <500 mg/kg, ●●● , >2000 mg/kg MON).

the best ML tree was −4232.95. Members of *F. avenaceum* and *F. acuminatum* including most isolates from Canada each form a well-supported clade. These are separated by *F. tricinctum* and

a number of lineages that represent individual isolates from other continents morphologically identified as *F. avenaceum* and *F. reticulatum*. Some of these isolates (BBA 67526, 69055, 71502) were previously characterized by Schütt[15] as potent producers of MON. A number of strains isolated from Canadian durum and barley formed a well-supported clade together with a culture (BBA 64698) of *F. flocciferum* from Syria. Together with members of *F. flocciferum* and *F. torulosum*, the basal clade of the tree included six more strains isolated from barley and silage in Canada. However, none of the species isolated from Harvest Program Samples in 2010 fell in the basal clade. Morphologically, these cultures fitted largely into the species concepts of *F. reticulatum* and *F. tricinctum*,[34] with sporodochial (macro)-conidia similar to *F. acuminatum* but generally three-septate, shorter, and with a less elongated apical cell. This lineage was closely related with two strains morphologically similar to *F. acuminatum* (BBA 70231, 70232) or *F. reticulatum* (BBA 71608). This indicates that certain morphological characters, such as the shape of macroconidia or colony characters, may show recurring patterns within distantly related groups as well.

The phylogenetic analysis based on DNA sequences of the two genetic markers revealed a rather simple population structure for isolates of *Fusarium avenaceum* and *F. acuminatum* from across western Canada (Figure 1). Individual lineages in the *F. acuminatum* clade did not receive significant bootstrap support (>70%), indicating that the group consists of a single phylogenetic species. In the *F. avenaceum* clade, one lineage comprising two isolates from CWRS in Alberta, one from rye in Manitoba, and several reference strains of *F. arthrosporioides* received moderate bootstrap support (72%). However, several other lineages were discovered in the *F. avenaceum* clade that did not receive significant bootstrap support. DNA sequences of additional genetic markers are needed to confirm the divergence of the lineages from the core *F. avenaceum* clade.

MON Method Evaluation. Table 2 summarizes results from an evaluation of the analytical method for the analysis of

Table 2. Mean ± Standard Deviation (*n* = 7) Percent Recoveries of MON from Fortified Cereal Grains

cereal grain	day	fortification concentration		
		0.030 mg/kg	0.20 mg/kg	2.0 mg/kg
wheat	1	90 ± 13%	101 ± 8%	95 ± 6%
	2	108 ± 15%	85 ± 5%	92 ± 5%
	3	89 ± 6%	85 ± 6%	91 ± 4%
	overall[a]	95 ± 15%	90 ± 10%	93 ± 5%
barley	1	107 ± 6%	87 ± 4%	94 ± 2%
oats	1	97 ± 12%	78 ± 10%	90 ± 2%

[a]*n* = 21 per fortification concentration.

MON in wheat. Blank wheat replicates (*n* = 7) were fortified with MON at three different concentrations and analyzed on three different days. A similar evaluation was performed with fortified barley and oats; however the analysis of *n* = 7 replicates fortified at three different concentrations was performed on only one day because there was no significant difference in results between these cereals and wheat during the validation.

Acceptable recoveries and precision were obtained at all fortification levels for all matrices when compared to method performance criteria for other mycotoxins present in grain.[35] Mean recoveries varied from 78% to 108%, and the relative standard deviations under repeatability conditions were all ≤16%. The limit of quantitation (LOQ) of 0.03 mg/kg was estimated

as the concentration producing a peak with a signal-to-noise ratio of 9:1.

Recoveries of MON from fortified blank samples analyzed throughout the study were repeatable and consistent with the data obtained during the method evaluation; the mean ± standard deviation percent recovery was 76 ± 8% (*n* = 14).

There are very little published data on the stability of MON in grains. Therefore, an in-house corn reference material that contained MON at 0.35 mg/kg was stored at room temperature and analyzed sporadically over 17 months. No trends suggesting an increase or decrease in the in-house reference material MON concentration were observed.

Mycotoxins in Cereals. In comparison to results from the analysis of durum from another study,[22] a summary of the results from the analysis of cereals for MON is provided in Figure 2. MON was measured the most frequently in wheat; 56% of wheat samples contained quantifiable levels of MON, as compared to 33% and 16% of rye and oat samples, respectively. However, MON was measured in these small cereal grains at a lower frequency than for CWAD, where 74% of samples contained MON.[22]

In addition to MON, only DON, HT-2, and T-2 were quantified in samples. All other analytes were below the LOQ of 0.05 mg/kg. DON was detected most often in oats (57% of samples), followed by wheat (48%) and then rye (33%). The distribution of DON results is summarized in Figure 2. HT-2 was measured in three samples: one each of rye, wheat, and oats. Concentrations in these three samples ranged from 70 to 180 μg/kg. T-2 was measured in only one sample. It was present at 50 μg/kg in the wheat sample that also contained 140 μg/kg HT-2.

■ DISCUSSION

Fungal Analysis of *Fusarium* Species Found on Wheat, Rye, and Oat. A number of *Fusarium* species can cause visual damage to plants and seeds of many cereal crops. By now, *F. graminearum* is perhaps the best known species of *Fusarium*, because it is considered the most important worldwide. This fact can be attributed to the impact it has on yield and grain quality, its ability to produce several different toxins including DON, and its abundance in Canada and in many other countries around the globe. In the present study, *F. graminearum* was recovered from a number of samples of all cereals studied, but its occurrence seemed to be more linked to visibly damaged kernels and not so much correlated with nonsymptomatic seeds. Therefore, it can be hypothesized that most of the DON detected in naturally contaminated wheat resulted from the presence of FDK caused by *F. graminearum*. This observation is in accordance with reports published previously.[36]

F. avenaceum is found worldwide, but occurs primarily in temperate climates. The host plant range of the species is broad, but *F. avenaceum* is commonly found on small-grain cereals, where the species is considered to be pathogenic.[37,38] The occurrence of *F. avenaceum* on cereals depends on various environmental and agronomic factors; the preeminence of more competitive species such as *F. graminearum* or *F. culmorum* is a particularly important factor. In addition, *F. avenaceum* is known to be able to contaminate grain with toxic secondary metabolites such as MON and ENNs. However, data from previous analytical surveys indicated that field conditions in Canada and northern Europe do not favor production of MON.[19,31] Data from the present study confirmed this hypothesis for samples of common wheat, oat, and rye. The prevalence of *F. avenaceum*
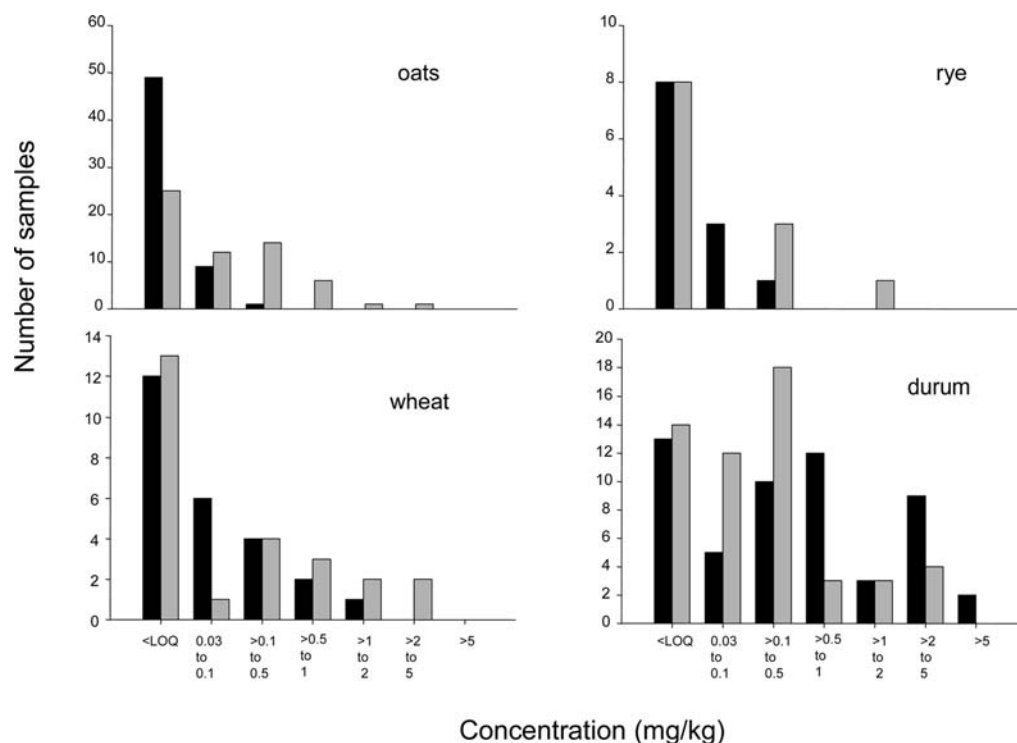
Figure 2. Distribution of moniliformin (black bars) and deoxynivalenol (gray bars) concentrations measured in small-grain cereal samples from the 2010 harvest. Data from durum are from Tittlemier et al.[22]

on durum wheat in 2010, however, resulted in significant contamination with MON and ENNs.[22] Another *Fusarium* species with a known propensity to form MON and ENNs on small-grain cereals is *F. acuminatum*. The species occurs worldwide on a range of substrates (e.g., soil, plants, insects). However, the species is considered only a weak pathogen of cereal grains and was reported from Canada only occasionally.[37,39] Therefore, any risk of MON contamination of Canadian grains by *F. acuminatum* can be considered comparatively low.

Compared to fungal analysis of fusarium-damaged kernels, frequencies of recovery of *F. poae* and *F. sporotrichioides* from random plated seeds are known to be higher in Canadian barley and oats.[40] At least one of the two *Fusarium* species was detected in all but two oats and in four rye of the present study's samples. Nonwheat cereals may be more sucseptible to infections by these species, which can lead to a higher risk of grain contamination with NIV and T-2/HT-2 toxins, respectively. *F. poae* and *F. sporotrichioides* are more often isolated from grain harvested under wet weather conditions,[19] a scenario that did not occur over much of the Canadian Prairies in the fall of 2010. This may also explain the fact that all samples of this study tested negative for contamination with NIV or T-2/HT-2 toxins.

Phylogenetic Species of *Fusarium* and Their Relationship to MON Producers. In several aspects, microscopic and cultural characteristics of *F. avenaceum* resemble those of *F. acuminatum* and *F. tricinctum*. The form of their sporodochial (macro)conidia and colony appearance in pure culture tend to look similar. The production of MON by *F. avenaceum* was first reported by Marasas et al.[41] and subsequently confirmed for all or at least most of the isolates tested by several others.[4,24,42] In the study of Schütt,[15] 11 out of 14 strains of *F. avenaceum* had the ability to produce moniliformin *in vitro*. From the three strains that did not produce MON, we included BBA 64135

isolated from canola in Germany in our study. In the phylogenetic analysis, the strain clustered with the majority of Canadian isolates from small cereals and other grains. However, together with an isolate of *F. avenaceum* from potato in Germany (BBA 64151) that produced significant amounts of MON *in vitro* and three other strains isolated from wheat in Canada and Italy, they formed a lineage within the *F. avenaceum* clade that was not strongly supported. Our data indicate that the propensity to form MON is a highly variable trait, as it was previously observed for the cryptic species of *F. subglutinans*.[11] Consequently, there is potential for MON contamination of small cereal grains in which *F. avenaceum* is present.

Other reference strains of the same species reported to produce significant amounts of MON in culture (BBA 67526, 189.5 mg/kg; BBA 69055, 2047.4 mg/kg)[15] did not fall in the *F. avenaceum* clade. In our phylogenetic analysis, each of these isolates represented an individual lineage that together formed a closely related sister clade to *F. avenaceum*. The cultures closely resembled *F. avenaceum* morphologically, but they were isolated from soil and citrus in warmer climates of Asia.

F. arthrosporioides is morphologically very similar to *F. avenaceum*, but can be distinguished by the color of aerial mycelia on filter paper after incubation in the dark. In addition, on SNA culture media with filter paper *F. avenaceum* tends to form pyriform (micro)conidia in the aerial mycelium. In our phylogenetic analysis, reference strains of *F. arthrosporioides* from colder climates formed a distinct lineage in the main *F. avenaceum* clade, which was previously reported from other studies as well.[43] The propensity to produce MON was observed in all isolates of *F. arthrosporioides* examined by Schütt.[15] Therefore, the formation of MON was reported to be species specific. Natural occurrence of *F. arthrosporioides* seems to be limited to temperate climates, where the species is known to be pathogenic on potatoes and other root crops.[37] These crops and

their produce are also at risk of being potentially contaminated with MON.

Morphological characters of *F. acuminatum*, such as the shape and size of sporodochial (macro)conidia, are similar to closely related species, such as *F. tricinctum* or *F. reticulatum*. All three species, however, can be distinguished by the type of (micro)condia in the aerial mycelium and differences in the formation of chlamydospores. Many isolates of *F. acuminatum* form chlamydospores in the substrate and a few allantoid microconidia in the aerial mycelium. When grown in darkness, microconidia of *F. tricinctum* often are citriform, but chlamydospores are rarely observed. This close relationship between *F. acuminatum* and *F. tricinctum* has been confirmed through phylogenetic studies, which also revealed several distinct phylogenetic lineages in the *F. tricinctum* clade.[44] In *F. reticulatum*, microconidia and chlamydospores are often not formed. Our analysis detected at least two disjunct lineages, the morphology of which resemble colony and microscopic characters of *F. reticulatum*. Canadian isolates from barley and silage, identified by P. E. Nelson as *F.* cf. *tricinctum* based on morphology, formed a sister clade to reference isolates from melon and maize in Turkey and Serbia, respectively. One of the Turkish isolates (BBA 70232) was reported to produce significant amounts of MON *in vitro*, whereas in another isolate from Turkey (BBA 70231) MON was not detected.[15] The variability of MON formation observed in strains from the same location and host was also reported for *Fusarium* isolates from *Medicago* seeds in South Africa.[20,45] In our phylogenetic analysis, an isolate from *Medicago* in South Africa (BBA 71502) represented a distinct phylogenetic lineage falling in between the *F. acuminatum* and *F. tricinctum* clades.

A number of Canadian strains isolated from barley, durum wheat, and oat in the late 1980s was grouped with a reference culture of *F. flocciferum* from barley in Syria. Occasionally, the species gets confused with *F. graminearum* because of similar morphological characters, but *F. flocciferum* has slightly smaller macroconidia and forms chlamydospores more abundantly. The species seems to fall into two major clades, one consisting of material from Europe (BBA 64346, 64365) and another mainly comprising isolates from North America. Canadian members of the *F. flocciferum* clade morphologically were more similar to *F. reticulatum*, forming smaller, more slender macroconidia. None of the seven strains of *F. flocciferum* studied by Schütt[15] produced MON *in vitro*.

MON in Cereals. There are very few data on the occurrence of MON in Canadian grain. In addition to recent work on durum grown in western Canada,[22] only two studies report on the natural occurrence of MON in Canada. Clear et al.[19] analyzed MON in red spring wheat composite samples prepared from CGC Harvest Survey Program samples obtained from the Canadian Prairies in 2002. MON was detected in only one out of 19 wheat composites; it was present at a concentration of 0.07 mg/kg. Scott and Lawrence[46] did not detect MON above the method limit of detection of 20 μg/kg in any of the eight western hard wheat or rye samples analyzed in their earlier study.

Most individual concentrations measured in the current study were greater than the 0.07 mg/kg reported for the one sample by Clear et al.[19] The median MON in wheat samples containing quantifiable concentrations was 0.19 mg/kg. Caution must be taken when comparing results from the current study to Clear et al.'s work though, because the act of compositing will dilute MON concentrations in individual samples. Therefore,

the higher concentrations observed in the individual samples from 2010 do not necessarily indicate MON was present at different concentrations than what was observed by Clear et al.[19] in 2002.

MON has been observed in small cereal grains in a number of northern European countries. Many of the concentrations measured in the 2010 Canadian samples are in the same range as those European MON concentrations recently reported in the literature. For example, MON concentrations reported for wheat from Norway from the 2000 through 2004 harvests ranged from <0.04 to 0.950 mg/kg.[31,47] MON concentrations in wheat from Germany and Finland were in similar ranges of <0.03 to 0.224 mg/kg and <0.02 to 0.810 mg/kg,[10,48] respectively. MON was also detected in the single oats sample obtained from Finnish harvests in 2001 and 2002 at 0.084 mg/kg.[10]

However, the concentrations measured in the 2010 Canadian samples are much less than the MON concentrations reported by Sharman[16] and Lew[49] in Polish wheat, rye, and oats that exhibited visible signs of fungal damage. These concentrations ranged from 0.5 to 25.2 mg/kg for wheat, 6.1 to 12.3 mg/kg for rye, and 15.7 to 38.3 mg/kg for oats.[16,49] The maximum concentrations observed in Canadian samples were 0.15, 0.13, and 1.87 mg/kg for oats, rye, and wheat, respectively.

Overall, MON was most relevant for wheat, in comparison to rye and oats. Figure 2 shows that the higher concentrations of MON were measured more often in wheat than the other two cereal grains, but less often than for CWAD.[22] Differences in MON concentration among various small cereal grains in Norway were also reported by Uhlig et al.[31] In a similar fashion to the current work, wheat had a greater tendency to contain quantifiable levels of MON as compared to oats and barley. The highest concentrations of MON for each harvest year were also found in wheat samples.

The occurrence of the small number of relatively high MON concentrations (i.e., over 0.5 mg/kg) observed in the 2010 Canadian samples is due, at least in part, to the atypical environmental conditions of the 2010 growing season.[22] Rainfall on the southern Canadian Prairies was greater than average from April to October 2010 [National Agroclimate Information Service, Agriculture and Agri-Food Canada; www.agr.gc.ca/drought]. The conditions on the Prairies during the 2010 growing season promoted the development of FHB in this area.

Other Fusarium-Produced Mycotoxins in Cereals. DON was often present in the oats, rye, and wheat samples analyzed in addition to MON. DON was more frequently observed in oats than MON, but otherwise these two mycotoxins occurred at similar frequencies in the samples. In general, higher DON concentrations were measured as compared to MON as well. This is illustrated in Figure 2 by the slight shift of DON concentration distributions to the right for oats, rye, and wheat.

The concentrations of DON detected in the 2010 samples are consistent with those reported in the literature for small cereal grains grown in Canada,[19,50] northern Europe,[47,51] and Argentina.[52] For example, the median DON concentration measured in wheat (0.65 mg/kg) matched the median concentration of DON detected in wheat (0.7 mg/kg) from the Canadian Prairies in 2000 through 2002.[19] The range of DON concentrations reported by Clear et al.[19] was greater than those observed in the current study though; the maximum concentration reported by Clear et al. was 7.9 mg/kg as compared to 4.0 mg/kg.

There are fewer data available to compare HT-2 and T-2 results, but the infrequent detection of these two mycotoxins at low concentrations was also noted for oats from southern Ontario, Canada.[50] In this study, HT-2 and T-2 were detected in only approximately 10% of fields sampled at concentrations of less than or equal to 0.5 mg/kg. These two mycotoxins were not detected in composite wheat samples from the Canadian Prairies studied in 2000 through 2002 though.[19]

The infrequent detection of HT-2 and T-2 in cereal grains from the Canadian Prairies, as well as the low concentrations
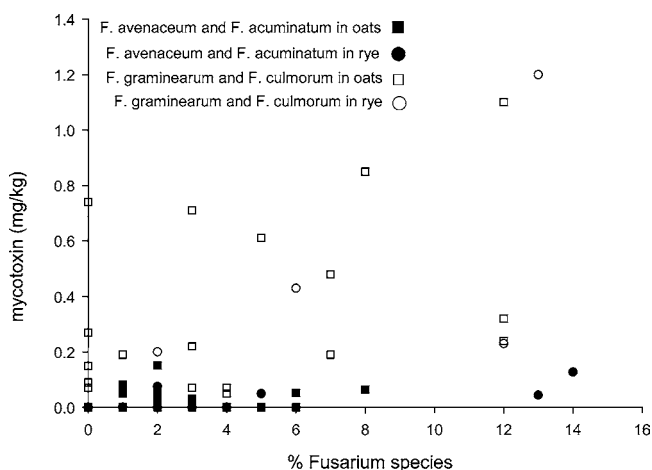
measured, contrasts with data from northern European countries. Maximum concentrations summarized by van der Fels-Kerx and Stratakou[53] from a number of European studies are up to 55 times higher than those observed in the current study.

Correlation between Mycotoxins and _Fusarium_ Species. The relationship between the presence of _Fusarium_ species and the mycotoxins they produce was examined in oats and rye samples (Figure 3), from which 100 randomly selected kernels were plated. There was no apparent relationship between MON and the percentage of kernels determined to contain either of these two MON-producing species (_F. avenaceum, F. acuminatum_) for oats and rye. This lack of a relationship is unexpected because these two species are known to produce MON and other groups have reported a relationship between the presence of _F. avenaceum_ and MON concentrations in cereal grains.[4,15] For example, Uhlig et al.[31] noted that MON concentrations were significantly correlated to the number of kernels infected with _F. avenaceum/ F. arthrosporioides_ and _F. culmorum_ in the Norwegian grain they analyzed. A statistically significant correlation was also reported between _F. avenaceum_ levels and concentrations of MON in Finnish wheat;[10] however the relationship did not hold for Finnish barley.

However, there was a tendency for DON concentration to increase with an increase in the percentage of kernels that contained _F. graminearum_ or _F. culmorum_. The relationship was a statistically significant linear regression (ANOVA, $p < 0.001$), with $R^2 = 0.42$. Such relationships have been noted previously.[54]

In the present study, the lack of relationship between MON contamination and percentage of seeds containing _F. avenaceum_ or _F. acuminatum_ may be explained through the degree of substrate colonization. Most of the kernels randomly selected

Figure 3. Relationship between the percentage of randomly selected plated kernels containing _Fusarium_ species and the associated concentrations of deoxynivalenol (white symbols) and moniliformin (black symbols) in the oats and rye samples.

Figure 4. Mean concentrations of deoxynivalenol (DON) and moniliformin (MON) in oat samples harvested in different crop districts across the Canadian Prairies in 2010. Results that were less than the limit of quantitation (LOQ) were set to 0 in order to calculate the mean.
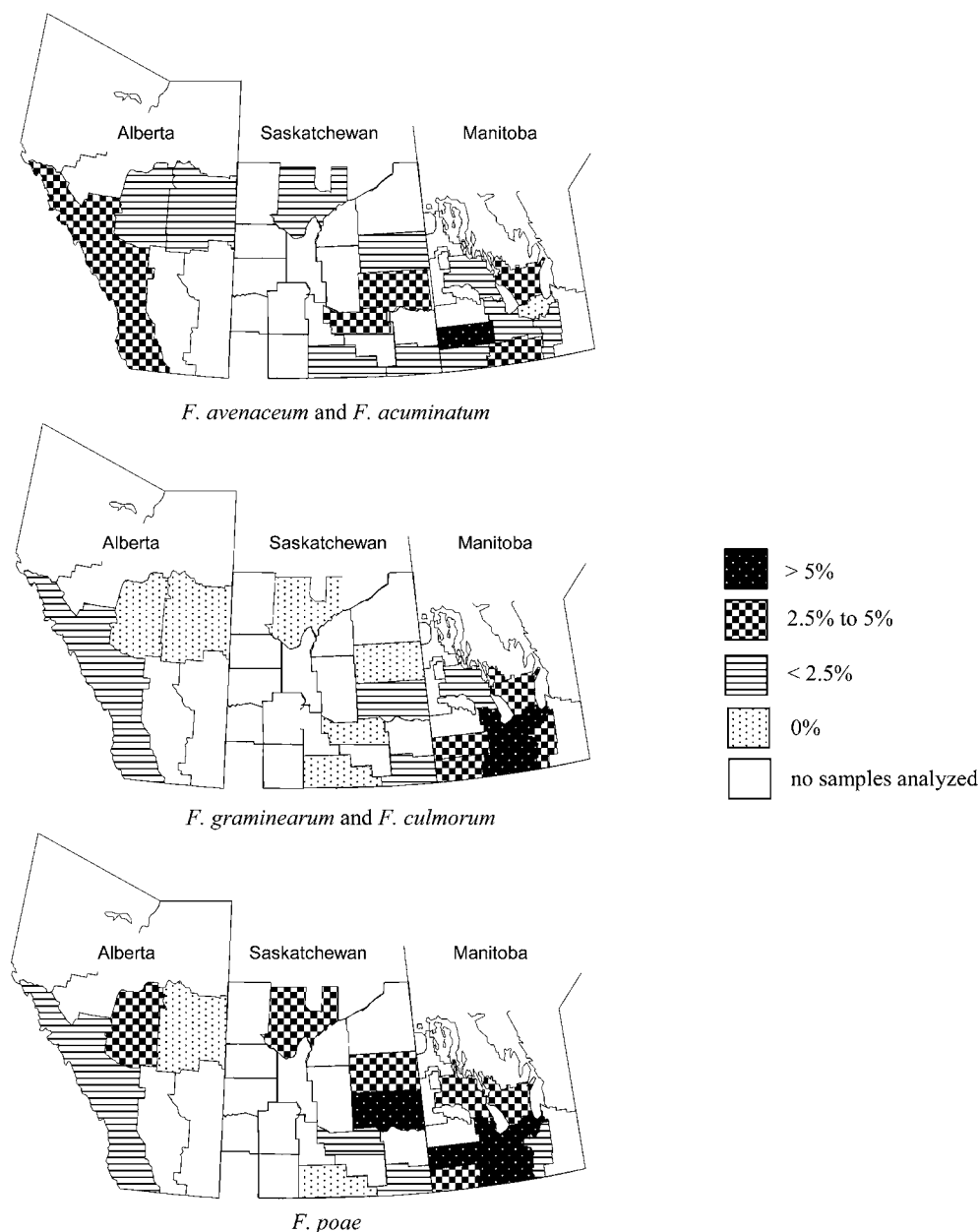
Figure 5. Mean frequency of detection of *Fusarium* species in randomly selected and plated kernels from oat samples harvested in different crop districts across the Canadian Prairies in 2010.

for agar plating probably were just lightly infected by fusaria. It is possible that the *Fusarium* species first need to exploit most of the nutrients from the endosperm before the MON biosynthesis pathway is up-regulated. For *Fusarium* species of section *Liseola* it is known that starvation can trigger or enhance fumonisin production in culture.[55,56] MON may play only a limited role as a pathogenicity factor during the infection process.

Geographical Distribution of Mycotoxin and *Fusarium* Species. The geographical distributions of mean MON and DON concentrations (Figure 4), as well as the occurrence of the respective fusaria that produce these two mycotoxins and *F. poae*, were determined for oats (Figure 5). This evaluation focused on oats because the most samples were available for this grain.

In general, *F. avenaceum* and/or *F. acuminatum* were observed the most frequently in oat samples from east central Saskatchewan and central Manitoba crop districts, as well as the most westerly crop district in Alberta (Figure 4). However,

mean MON concentrations were highest in central Manitoba crop districts, as well as one southwestern Manitoba and one east central Saskatchewan crop district. The lack of consistency between the crop districts from which the samples with the highest percentage of kernels containing *F. avenaceum* and/or *F. acuminatum* and those with the highest concentrations of MON originated is consistent with the lack of relationship observed in Figure 3.

There appears to be more consistency between crop districts where samples contained relatively high concentrations of DON and a high frequency of detection of *F. graminearum* and/or *F. culmorum* in oats. Samples originating from south central Manitoba, as well as southeastern Saskatchewan, had the highest frequency of *F. graminearum* and *F. culmorum* detection. This apparent gradient of decrease from east to west was also displayed in the mean DON concentrations; however, samples from two crop districts in Alberta contained comparable

concentrations to districts in central Manitoba. There are no data on DON in oats from past harvests on the Canadian Prairies for comparison; however a similar decrease in DON measured on an east to west gradient across the Prairies was noted for wheat in the early 2000s.[19]

The geographical distribution of the frequency of detection of *F. poae* was similar to that of *F. graminearum* and/or *F. culmorum*. There was however some more northerly crop districts in Saskatchewan and Alberta from where samples contained more *F. poae*. Nivalenol was not detected in any samples though. In conclusion, phylogenetic analyses of DNA sequence data presented here revealed a rather simple population structure of *F. avenaceum* and *F. acuminatum*, the two most important *Fusarium* species known in the Canadian Prairies to contaminate small cereal grains with MON. For the major phylogenetic lineages, we confirmed that the propensity to form MON is a highly variable trait in the phenotype of individual strains. Canadian isolates of *F. avenaceum* and *F. acuminatum* from cereals and other grains grouped all with the major lineage of these species, respectively. However, some reference cultures isolated from other continents represent distinct phylogenetic lineages that fall in between major clades. Members of these minor lineages are morphologically similar to *F. avenaceum* or closely related species and show potential to produce significant amounts of MON *in vitro* and *in planta*.

Moniliformin and MON-producing *Fusarium* species were observed in many of the common wheat, oat, and rye samples analyzed from the 2010 harvest. However, the occurrence and frequency of detection were not as high as observed for durum wheat obtained from the same harvest.[22] DON and the associated *F. graminearum* and *F. culmorum* were observed more frequently in the small cereal grains analyzed in this study, as compared to MON and *F. avenaceum* and *F. acuminatum*.

There was limited evidence for a strong relationship between MON and the presence of MON-producing *Fusarium* species in the samples analyzed in this study. There was no apparent relationship between MON concentrations and *F. avenaceum* and *F. acuminatum* in rye and oat samples, and the geographical distribution of MON and *F. avenaceum* and *F. acuminatum* across the Canadian Prairies displayed minor similarities. The absence of a clear relationship between MON and MON-producing *Fusarium* species in common wheat, rye, and oats differs from what was observed for durum obtained from the same harvest[22] and may reflect substrate or microbiome differences.

ASSOCIATED CONTENT

Ⓢ Supporting Information

This information is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Author Contributions

T.G. and S.A.T. organized the research project and shared in the data analysis and writing. M.R., R.T., D.G., and J.M.C. were responsible for managing and performing the mycotoxin analyses.

S.K.P. and R.M.C. performed the fungal analysis. T.M. is responsible for the Canadian Grain Commission Harvest Sample Program.

Notes

The authors declare no competing financial interest.

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