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Nivalenol-producing *Fusarium cerealis* associated with fusarium head blight in winter wheat in Manitoba, Canada

C. C. Amarasinghe[a], S. A. Tittlemier[b] and W. G. D. Fernando[a]*

[a]*Department of Plant Science, University of Manitoba, Winnipeg, MB, R3T 2N2; and* [b]*Grain Research Laboratory, Canadian Grain Commission, Winnipeg, MB, R3C 3G8, Canada*

Fusarium head blight (FHB) in small grain cereals is primarily caused by the members of the *Fusarium graminearum* species complex. These produce mycotoxins in infected grains, primarily deoxynivalenol (DON); acetylated derivatives of DON, 3-acetyl-DON (3-ADON) and 15-acetyl-DON (15-ADON); and nivalenol (NIV). This study reports the isolation of *Fusarium cerealis* in infected winter wheat heads for the first time in Canada. A phylogenetic analysis based on the *TRI101* gene and *F. graminearum* species-specific primers revealed two species of *Fusarium*: *F. graminearum sensu stricto* (127 isolates) and *F. cerealis* (five isolates). Chemotype determination based on the *TRI3* gene revealed that 65% of the isolates were 3-ADON, 31% were 15-ADON and 4% were NIV producers. All the *F. cerealis* isolates were of NIV chemotype. *Fusarium cerealis* isolates can often be misidentified as *F. graminearum* as the morphological characteristics are similar. Although the cultural and macroconidial characteristics of *F. graminearum* and *F. cerealis* isolates were similar, the aggressiveness of these isolates on susceptible wheat cultivar Roblin and moderately resistant cultivar Carberry differed significantly. The *F. graminearum* 3-ADON isolates were most aggressive, followed by *F. graminearum* 15-ADON and *F. cerealis* NIV isolates. The findings from this study confirm the continuous shift of chemotypes from 15-ADON to 3-ADON in North America. In Canada, the presence of NIV is limited to barley samples and the discovery of NIV-producing *F. cerealis* species in Canadian wheat fields may pose a serious concern to the Canadian wheat industry in the future.

Keywords: chemotypes, deoxynivalenol, *Fusarium cerealis*, *Fusarium graminearum*, fusarium head blight, nivalenol

Introduction

Fusarium head blight (FHB), also called scab, is an economically important fungal disease in many crops including wheat, barley and oats (Parry *et al.*, 1995). Although many species of *Fusarium* contribute to this disease, *Fusarium graminearum* (teleomorph *Gibberella zeae*) is considered to be the major pathogen of FHB in many countries (Parry *et al.*, 1995; McMullen *et al.*, 1997). *Fusarium culmorum*, *Fusarium avenaceum*, *Microdochium nivale*, *Fusarium verticilloides*, *Fusarium oxysporum* and *Fusarium poae* are the other related species that play a minor role in FHB development (Parry *et al.*, 1995; Liddell, 2003). These fungi, in association with FHB in cereals, produce mycotoxins that lead to contamination of grains posing a concern to the cereal industry. For example, *Fusarium* infection alters the physical properties of wheat kernels and therefore causes a detrimental effect on the processing quality of wheat.

Fusarium cerealis (syn. *Fusarium crookwellense*) is an important pathogen that causes root rot and seedling blight of cereals. *Fusarium cerealis* has been reported in North America, South Africa, Australia, New Zealand, China, Japan and European countries (De Nijs *et al.*, 1996; Tan *et al.*, 2004 Šrobárová *et al.*, 2008). In Europe, *F. cerealis* was often found within the FHB pathogen complex causing red rot in corn (Logrieco *et al.*, 2003). *Fusarium cerealis* is reported to be the second most widespread pathogen on corn heads and leaves in New Zealand (Lauren & Minna, 1999). Sugiura *et al.* (1994) reported the presence of *F. cerealis* in the complex of wheat blight pathogens in Japan. Recently, Schmale *et al.* (2011) have reported on one isolate of *F. cerealis* found in wheat fields in eastern USA.

The type B trichothecene mycotoxins deoxynivalenol (DON) and nivalenol (NIV) are the main mycotoxins found in *Fusarium*-infected wheat kernels. *Fusarium graminearum*, *F. culmorum* and *F. cerealis* isolates produce type B trichothecenes. Although *F. graminearum* isolates produce DON and its acetylated derivatives 3-acetyl-DON (3-ADON) and 15-acetyl-DON (15-ADON), and NIV, no single *F. graminearum* isolate is known to produce both DON and NIV (Goswami & Kistler, 2005). Unlike *F. graminearum*, *F. cerealis* isolates produce NIV and not DON (Ichinoe *et al.*, 1983). It has been reported that *F. cerealis* may also produce fusaric acid, zearalenone, fusarin C and diacetoxyscirpenol (Bacon *et al.*, 1996; Bottalico & Perrone, 2002).

In Canada, DON is the primary mycotoxin in *Fusarium*-infected grain (McMullen *et al.*, 1997), but in Asia

*E-mail: dilantha.fernando@umanitoba.ca

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and Europe both DON and NIV are common contaminants of grain (Ichinoe *et al.*, 1983). The acetylated derivatives of DON, 3-ADON and 15-ADON, increase the total DON contamination in wheat. Although NIV is less pathogenic to plants, it has been reported that NIV is more toxic to animals than DON (Ueno, 1977; Visconti *et al.*, 1991). Both DON and NIV pose a significant threat to the human and animal systems by inhibiting the protein synthesis process.

Molecular techniques have allowed the differentiation of *F. graminearum* from other species (Demeke *et al.*, 2005) and provided genetic information on the species populations (Cumagun *et al.*, 2004; Fernando *et al.*, 2006). PCR assays have also been developed to characterize the different chemotypes (3-ADON, 15-ADON, NIV) of *Fusarium* spp. (Guo *et al.*, 2008; Ward *et al.*, 2008). These molecular techniques help to identify the type of mycotoxins produced by different *Fusarium* spp. and their genetic diversity. Lee *et al.* (2002) reported the importance of the *TRI13* gene in the *TRI5* cluster. The *TRI13* gene is responsible for regulating the DON–NIV switch in *Fusarium* isolates. High-throughput multilocus genotyping has been extensively used in *Fusarium* species identification. One of the major genes used in species identification is *TRI101* (O'Donnell *et al.*, 2004; Starkey *et al.*, 2007). This gene encodes a trichothecene 3-*O*-acetyltransferase that transfers an acetyl group from acetyl-CoA to the C-3 hydroxyl moiety of the trichothecene molecule (Garvey *et al.*, 2008). Disruption of *TRI101* in *Fusarium sporotrichioides* blocks the production of T-2 toxin, a trichothecene-derived metabolite, and leads to the accumulation of an intermediate, isotrichodermol, suggesting that the *TRI101* gene product is necessary for the production of trichothecenes (McCormick *et al.*, 1999).

To the best of the authors' knowledge, NIV-producing *F. graminearum* isolates have not yet been reported in *Fusarium*-infected wheat fields in Canada. Gale *et al.* (2011) reported that populations of *F. graminearum* collected from infected wheat heads in Louisiana were mainly composed of NIV-producing isolates. However, in Canada testing for NIV is not routinely conducted at mills or elevators. Therefore, the discovery of *Fusarium* spp. that are capable of producing NIV may cause increasing concern to the wheat industry in Canada. This study reports the presence of NIV-producing *F. cerealis* in infected winter wheat plots in Manitoba, Canada, for the first time. The objectives of this study were: (i) to identify *Fusarium* spp. in winter wheat plots in Carman, Manitoba, Canada; (ii) to determine the chemotype diversity of *Fusarium* spp.; and (iii) to characterize the ability of these *Fusarium* spp. to induce FHB on wheat in greenhouse pathogenicity experiments.

Materials and methods

Sample collection and isolation

Wheat heads with symptoms were collected from seven naturally infected winter wheat varieties, Buteo, Accipiter, Falcon, McClintock, Moats, 39M*11 and Readymade, grown at the Ian Morrison Research Farm, Carman, Manitoba, Canada in 2013. Wheat heads with top, middle and bottom infection as well as full head and peduncle infection were collected separately, placed in paper bags and transported in cooler boxes to Winnipeg, Manitoba. Samples were stored at −20°C until processing. Isolations were done from each head. Infected seeds were surface disinfected in 1% sodium hypochlorite for 1 min and air dried on sterile filter paper. Seeds were plated individually on potato dextrose agar (PDA; Difco Laboratories) and incubated at 25°C for 4–7 days under fluorescent light. As *Fusarium* spp. grew out of the seeds, colonies were chosen and subcultured to obtain pure cultures. All isolates were then plated onto *Fusarium*-specific nutrient-poor Spezieller Nährstoffarmer agar (SNA) plates in order for sporulation to occur. Sporodochia in the SNA medium were washed using 50 μL sterile distilled water, and the macroconidia suspension was spread over water agar (WA) plates. These plates were incubated under the same conditions for 4–6 h and a single germinating macroconidium was transferred onto PDA plates and incubated for 5–7 days. These single spore isolates were preserved until further use.

DNA extraction

Single spore isolates were placed on PDA and incubated as described above. Once plates were covered with fungal growth, mycelia were harvested, lyophilized and stored at −20°C until further use. DNA extraction was carried out according to Fernando *et al.* (2006). Briefly, the lyophilized mycelium was broken into smaller pieces using a sterile toothpick followed by grinding in 600 μL TES buffer (100 mM Tris, 10 mM EDTA, 2% sodium dodecyl sulphate) in a 1·5 mL microcentrifuge tube using a pellet pestle. After this, 140 μL 5 M NaCl and 70 μL 10% cetyltrimethylammonium bromide (CTAB) were added to the tube and vortexed. The mixture was incubated at 65°C for 20 min. Following incubation, 600 μL phenol:chloroform:isoamyl alcohol (25:24:1 v/v) was added and then centrifuged at 9000 ***g*** for 15 min. The supernatant was transferred into a new tube and the latter step was repeated. DNA was precipitated by adding 80 μL 5 M NaCl and 1 mL 100% ethanol, followed by centrifugation at 12 000 ***g*** for 5 min. The DNA pellet was washed with 200 μL ice-cold 80% ethanol. After air-drying, the pellet was suspended in 400 μL warm sterile water (65°C). Following full resuspension, DNA was treated with RNase (0·75% v/v) and stored at −20°C until further use.

Identification of isolates to species

Isolates were first amplified using *F. graminearum* species-specific PCR primers described by Demeke *et al.* (2005). Two primers were used in the PCR, Fg16F (5′-CTCCGGATATGTTGCGT CAA-3′) and Fg16R (5′-GGTAGGTATCCGACATGGCAA-3′), which amplify a fragment of 450 bp. PCR was performed in a 25 μL reaction volume containing 20 ng template DNA, 1·5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris.HCl (pH 8·0), 0·2 mM of each dNTP, 0·4 μM of each primer, and 0·75 U *Taq* DNA polymerase. The PCR amplification protocol consisted of an initial denaturation at 95°C for 3 min; followed by 35 cycles of 30 s at 95°C, 1 min at 56·7°C, 1 min at 72°C; and a final extension of 72°C for 5 min. PCR products were run on a 1% agarose gel.

To identify isolates that did not amplify with *F. graminearum* species-specific primers, a portion of the *TRI101* gene was amplified and bidirectionally sequenced. The primers used for amplification were TRI101F (5′-CCATGGGTCGCRGGCCARGTS AA-3′) and TRI101R (5′-AACTCSCCRTCIGGYTTYTTNGG

CAT-3′) (Proctor *et al.*, 2009). PCR was performed in a 25 μL reaction volume as described above. The PCR amplification protocol consisted of an initial denaturation at 95°C for 2 min; followed by 30 cycles of 40 s at 95°C, 30 s at 54°C, 90 s at 72°C; and a final extension of 72°C for 7 min. For sequence analysis, PCR-amplified DNA fragments were purified with Exosap-IT PCR product clean-up kit (Affymetrix), according to manufacturer's instructions. Sequencing reactions were carried out using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems), with reaction products analysed on an Applied Biosystems 3730xl DNA Analyzer at Eurofins MWG Operon. Sequences were assembled, trimmed and edited using GENEIOUS v. 5.4.5 (Drummond *et al.*, 2011). The sequences of the PCR products were aligned manually using BIOEDIT v. 7.1.3 sequence alignment editor (Hall, 1999). The final data set had an aligned length of 1208 bp. For estimating the appropriate model of sequence evolution, a hierarchical likelihood ratio test (hLRT) was carried out using MODELTEST v. 3.8 (Posada & Crandall, 1998). The hLRT criterion indicates that the HYM + G (Tamura & Nei, 1993) represent the optimal model for the data set. Posterior probability (PP) distributions of trees were created using the Markov chain Monte Carlo (MCMC) method and following search strategies suggested by Huelsenbeck *et al.* (2002). Four chains were run simultaneously (10 000 000 generations), starting from random trees. Chains were collected every 1000 generations and the respective trees were written to a tree file. All runs reached a plateau in log likelihood score, which was indicated by the standard deviation of split frequencies (0·02), and the potential scale reduction factor was close to 1, indicating that the four MCMC chains converged. The initial 2500 trees were discarded as burn-in before stationary was reached. The 50% majority rule consensus tree was developed from the remaining 7500 trees. Calculation of the consensus tree and of the posterior probability of clades was done based upon the trees sampled after the burn-in, and trees were compiled and drawn using FIGTREE v. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). Sequences of reference isolates from the ARS (NRRL) Culture Collection were downloaded from GenBank and used in the alignment for species identification. *Fusarium pseudograminearum* was used as the out-group.

Determination of chemotypes using PCR assay

Chemotype identity of each isolate was determined using multiplex PCR primers 3CON, 3NA, 3D3A and 3D15A (Ward *et al.*, 2002). The multiplex PCR primers generate a 840 bp fragment from NIV-producing strains, a 610 bp fragment from 15-ADON producers and a 243 bp fragment from 3-ADON producers. PCR was performed in a 15 μL volume containing 20 ng template DNA, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris.HCl (pH 8·0), 0·2 mM each dNTP, 0·4 μM each primer, and 0·75 U *Taq* DNA polymerase. PCR cycling conditions consisted of an initial denaturation at 94°C for 4 min; followed by 35 cycles of 1 min at 94°C, 40 s at 58°C, 40 s at 72°C; and a final extension of 72°C for 6 min. PCR amplicons were separated on a 2% agarose gel.

Analysis of colony morphology and macroconidia of *F. graminearum* and *F. cerealis*

To determine the cultural characteristics of *F. cerealis* and *F. graminearum*, isolates were grown on PDA for 1 week in the dark at 22–23°C. The macroconidia were produced in SNA nutrient-deficient medium and observed under a microscope (Leica Microsystems Inc.) using LEICA APPLICATION SUITE v. 2.8.1.

Aggressiveness experiments and statistical analysis

Fusarium cerealis and *F. graminearum* isolates were individually inoculated on the susceptible wheat cultivar Roblin and moderately resistant wheat cultivar Carberry. Three isolates each of *F. graminearum* 15-ADON, *F. graminearum* 3-ADON and *F. cerealis* NIV strains were used in this study. Wheat plants were grown in plastic pots containing Sunshine Mix. Plants were fertilized every 2 weeks with NPK (20:20:20). The plants were arranged in a completely randomized design with 10 replicates. *Fusarium* inoculations were done by point inoculations. Three to four spikes were inoculated per plant, once individual spikes were close to 50% anthesis. Two florets in a spikelet were inoculated by injecting 10 μL of a macroconidial suspension adjusted to 5×10^4 spores mL^{-1} between the lemma and palea of a floret. Following inoculation, a glassine bag was placed over the spike to increase humidity. The bags were removed 48 h after inoculation. Disease severity was rated 7, 10, 14 and 21 days after inoculation. Disease severity was rated using the FHB disease scale (Stack & McMullen, 1995). Disease severity measured the average percentage of spikes that were infected, on a scale of 0 (indicating no infection) to 100% (indicating completely infected spikes).

Analysis of nivalenol in *F. cerealis* infected wheat samples

In order to confirm the production of NIV by *F. cerealis* isolates, the infected wheat kernels were subjected to GC-MS analysis. Wheat kernels from 10 replicated heads of each isolate were pooled, ground and analysed by GC-MS according to the protocol described by Tittlemier *et al.* (2013). Eight *Fusarium* trichothecenes: DON, 3-ADON, 15-ADON, NIV, T-2 toxin, and its conversion product HT-2 toxin, diacetoxyscirpenol (DAS) and fusareon-X (FUS-X), were included in the GC-MS analysis, each with a quantitation limit of 0·05 mg kg^{-1}.

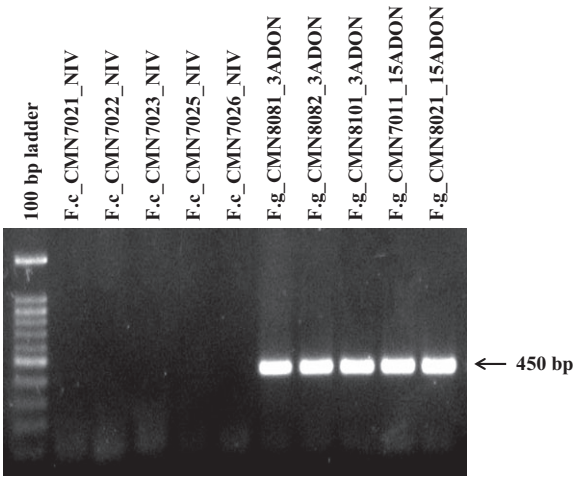

Figure 1 Representative PCR amplicons for Fg16F/R *Fusarium graminearum* species-specific primers. PCR products of 450 bp are produced by *F. graminearum* isolates. Representative isolates of *Fusarium cerealis* NIV (lanes 2–6), did not give any amplification for Fg16F/R primers. *Fusarium graminearum* 3-ADON (lanes 7–9) and *F. graminearum* 15-ADON (lanes 10–11) gave the amplified product of 450 bp.

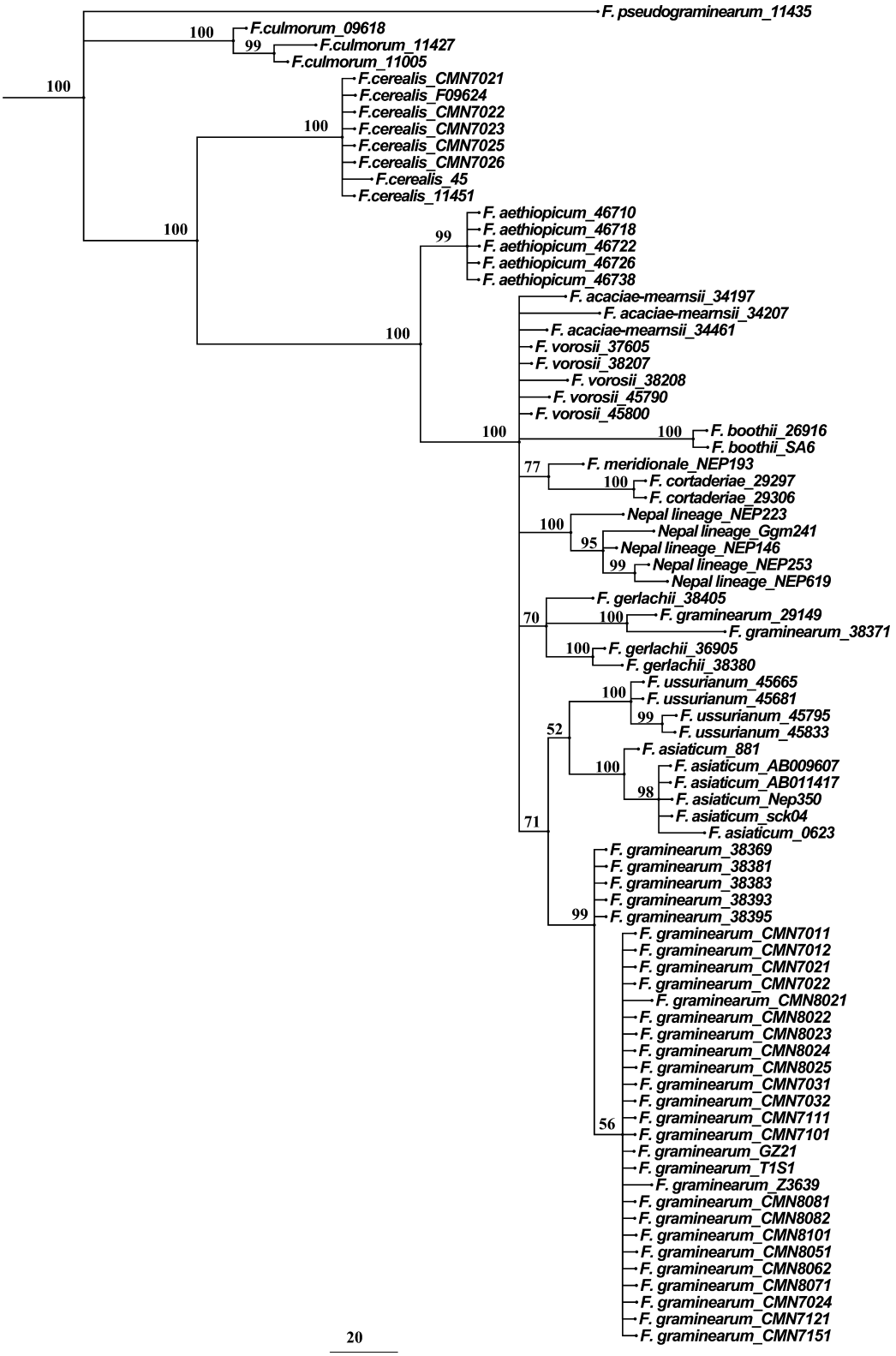

Figure 2 Phylogenetic tree based on a total of 1208 bp of *TRI101* gene sequences derived from Bayesian analysis. Representative isolates of *Fusarium graminearum* (22) and *Fusarium cerealis* (5) are shown in the figure by _CMN strain code name. Other representative isolates from different *Fusarium* spp. were downloaded from GenBank. NRRL strain designations and strain codes follow the underscore after the species name. Numbers at the nodes represent the posterior probability values derived from the Bayesian analysis.

Results

Fusarium species identification was done by PCR using *F. graminearum* species-specific primers Fg16F/R and also by sequencing a portion of the *TRI101* gene. Based on the Fg16F/R species-specific PCR assay, a 450 bp fragment characteristic of *F. graminearum* was obtained in 127 isolates. Thus, these 127 isolates were identified as *F. graminearum* (Fig. 1). However, five isolates did not show the expected 450 bp fragment. Therefore, these five isolates, along with 22 isolates randomly selected from the 127 PCR-positive isolates for Fg16F/R species-specific primers, were sequenced based on the *TRI101* gene. Partial sequences of the *TRI101* gene were used to identify the five unknown isolates to species level.

A phylogenetic tree based on 1208 bp of the *TRI101* gene sequence revealed three distinct clades (Fig. 2). These three clades represent *F. culmorum*, *F. cerealis* and *F. graminearum*. The five non-*Fusarium graminearum* isolates (herein called unknown) in the collection (CMN7021, CMN7022, CMN7023, CMN7025, CMN7026) clustered with the known *F. cerealis* isolates downloaded from GenBank (*F. cerealis* isolate 45, *F. cerealis* NRRL 11451 and *F. cerealis* isolate F09624). The unknown isolates formed a distinct monophyletic clade with the known *F. cerealis* isolates with 100% posterior probability. This analysis confirms that the unknown *Fusarium* isolates belong to the *F. cerealis* clade. These *F. cerealis* isolates were recovered from the winter wheat cv. Acciptor. The other 22 *F. graminearum* isolates that are already identified from the Fg16F/R PCR assay formed a monophyletic clade with other *F. graminearum* isolates downloaded from GenBank (GZ21, T1S1, Z3639; Fig. 2). These isolates did not cluster with other species in the *F. graminearum* species complex. This shows that they represent *F. graminearum sensu stricto* or lineage 7, which is commonly distributed in Canada and North America.

The trichothecene chemotypes of all 127 *F. graminearum* and five *F. cerealis* isolates were evaluated by using a multiplex PCR assay based on the *TRI3* gene (Fig. 3). According to the multiplex PCR assay, 65% of the isolates were 3-ADON and only 31% were 15-ADON. Five isolates showed the amplified band for NIV, which represented 4% of the total isolates. All the NIV-producing isolates were found to be *F. cerealis*.

The morphological and cultural characteristics of *F. graminearum* and *F. cerealis* were also compared. Colonies of both *F. graminearum* and *F. cerealis* grew rapidly, with flocculent aerial mycelia. Although *F. graminearum* mycelia were fuzzy with white-pink or pink colouration, mycelia of *F. cerealis* were fuzzy with a red-pink colour. The reverse colour of *F. graminearum* was more pinkish than the *F. cerealis* isolates. Macroconidia of *F. cerealis* were sickle-shaped, thick-walled with dorsoventral side significantly curved compared to the ventral side. Although most *F. cerealis* macroconidia had five septa, a few had four septa (Fig. 4b). Similar to *F. cerealis* macroconidia, *F. graminearum* macroconidia

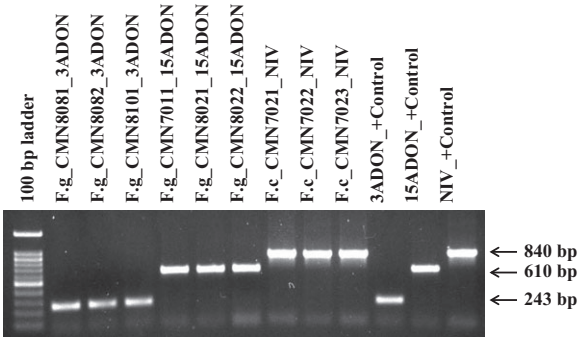

Figure 3 Representative PCR amplicons for multiplex PCR assay. PCR products of 243, 610 and 840 bp are produced for 3-ADON, 15-ADON and NIV isolates, respectively. Representative isolates of *Fusarium graminearum* 3-ADON (lanes 2–4), *F. graminearum* 15-ADON (lanes 5–7) and *Fusarium cerealis* NIV (lanes 8–10) are shown. Positive controls of 3-ADON, 15-ADON and NIV isolates are shown in lanes 11–13, respectively.

were also sickle-shaped but the curve of *F. graminearum* macroconidia on the dorsoventral side was less than on the ventral side. In addition, most *F. graminearum* macroconidia had five septa and some had three or four septa (Fig. 4a).

Finally, pathogenicity tests of *F. cerealis* and *F. graminearum* were conducted on susceptible (S) and moderately resistant (MR) wheat cultivars and it was found that the aggressiveness of *F. cerealis* was lower than that of *F. graminearum* on both S and MR wheat cultivars. *Fusarium graminearum* isolates showed disease symptoms 5 days post-inoculation (dpi) whereas *F. cerealis* took 7 days to show symptoms in S cv. Roblin and 10–12 days in MR cv. Carberry. Highest disease severity on both cvs Roblin and Carberry was shown by 3-ADON isolates followed by 15-ADON and NIV isolates. Significant differences for disease severity were also observed among the *Fusarium* isolates at both 14 dpi (Fig. 5) and 21 dpi (Fig. 6). Data from GC-MS also confirmed the production of NIV by *F. cerealis* isolates. The examined *F. cerealis* isolates produced NIV in infected grains of S and MR wheat cultivars at levels of 1·39–6·15 and 0·66–0·99 mg kg^{-1}, respectively. The other examined trichothecenes (DON, 3-ADON, 15-ADON, NIV, T-2 toxin, HT-2 toxin, DAS and FUS-X) were not detected in the analysed grain samples.

Discussion

To the best of the authors' knowledge, this study provides the first evidence on the presence of NIV-producing *F. cerealis* isolates in winter wheat fields in Carman, Manitoba, Canada. Miller *et al.* (1991) have reported the presence of two *F. crookwellense* isolates in Ontario, Canada. Since then, there have been no other reports on *F. cerealis* isolates from Canada. Recently, a single NIV-producing *F. cerealis* isolate was identified in New York (Schmale *et al.*, 2011). Zhang *et al.* (2011) and Castan-

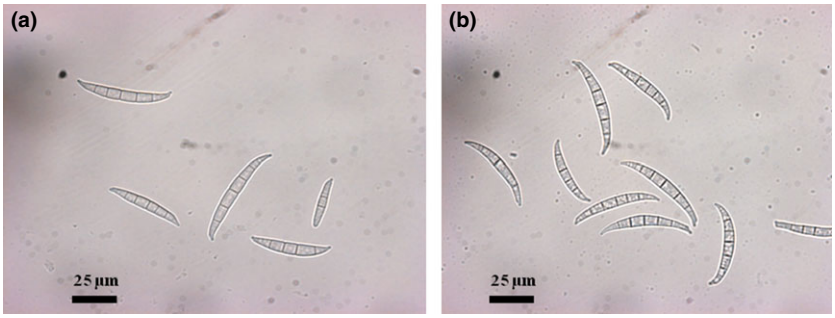

Figure 4 Macroconidia of *Fusarium graminearum* (a) and *Fusarium cerealis* (b) on Spezieller Nährstoffarmer agar medium.

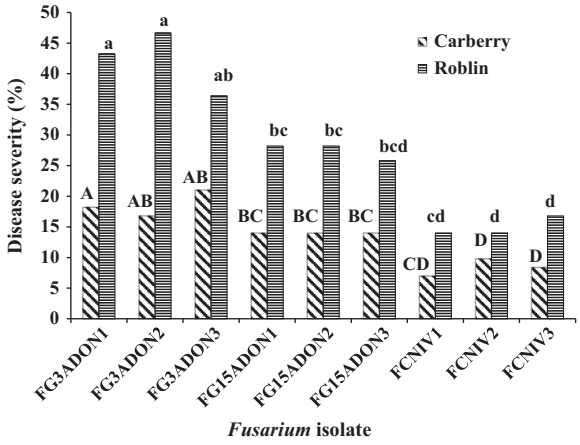

Figure 5 Mean fusarium head blight disease severity of wheat cultivars Carberry and Roblin at 14 days after inoculation with *Fusarium graminearum* 3-ADON isolates, 15-ADON isolates and *Fusarium cerealis* NIV-producing isolates. Means with the same letter for disease severity are not significantly different.

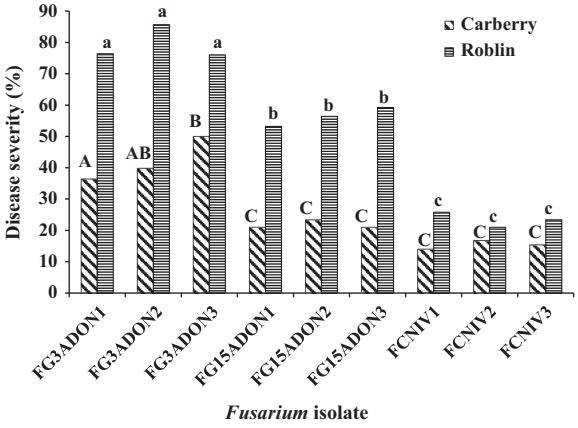

Figure 6 Mean fusarium head blight disease severity of wheat cultivars Carberry and Roblin at 21 days after inoculation with *Fusarium graminearum* 3-ADON isolates, 15-ADON isolates and *Fusarium cerealis* NIV-producing isolates. Means with the same letter for disease severity are not significantly different.

ares *et al.* (2013) also reported the presence of *F. cerealis* in barley seeds. Such recent reports on the occurrence of *F. cerealis* raises concern on the emergence of *F. cerealis* as one of the major pathogens causing FHB. *Fusarium cerealis* and *F. graminearum* are morphologically very similar and difficult to distinguish from each other in commonly used media, with their macroconidia having a similar shape (Yli-Mattila & Gagkaeva, 2010). Several studies reported that *F. cerealis* macroconidia are stout, thick-walled, with curved apical and basal cells and usually 5-septate. Similarly, *F. graminearum* macroconidia were sickle-shaped, elliptically curved and have five septa (Sugiura *et al.*, 1994; Yli-Mattila & Gagkaeva, 2010). Therefore, molecular techniques have been more effective in identifying these two species.

This study used *TRI101* sequences of *F. graminearum* species complex, *F. culmorum*, *F. cerealis* and *F. pseudograminearum* (as the out-group) from GenBank for phylogenetic analysis. *Fusarium graminearum* species complex included *F. aetheiopicum*, *F. acacia-mearnsii*, *F. vorosii*, *F. cortadariae*, *F. meridionale*, *F. boothii*, Nepal lineage, *F. gerlachii*, *F. ussurianum*, *F. asiaticum*, *F. graminearum sensu stricto* and novel *F. graminearum* isolates from the Louisiana Gulf Coast population. *Fusarium graminearum* isolates collected from winter wheat plots in Carman, Manitoba, Canada, formed a distinct cluster with other *F. graminearum s.s.* isolates obtained from GenBank. The *F. cerealis* isolates identified in this study formed a distinct cluster with known *F. cerealis* isolates from GenBank. These analyses of *TRI101* gene sequences confirmed the presence of *F. cerealis* in infected wheat heads collected from winter wheat plots in Carman, Manitoba, Canada.

All the *F. cerealis* isolates in this study were of NIV chemotype. This finding was similar to the reports from Germany, Poland, Japan and Russia (Sugiura *et al.*, 1994; Chandler *et al.*, 2003). Other reports indicate that *F. cerealis* is also capable of producing the mycotoxin zearalenone in infected barley seeds (Di Menna *et al.*, 1991; Miller *et al.*, 1991). Until 2012, the presence of NIV chemotypes in Canadian grain samples was limited to barley samples (Tittlemier *et al.*, 2013); no NIV chemotypes have been found in infected wheat samples. Therefore, the finding of NIV-producing *F. cerealis* isolates in wheat fields in Carman, Manitoba, Canada, comes as a serious concern for the wheat industry in Canada. A chemotypic shift from 15-ADON isolates to

3-ADON isolates was also observed in this study, as reported by Ward *et al.* (2008) and Guo *et al.* (2008) in western Canada. Specifically, of the 132 *Fusarium* isolates studied, 65% were 3-ADON and 31% were 15-ADON isolates.

The pathogenicity tests revealed that *F. cerealis* isolates were able to cause FHB in wheat but were less aggressive than *F. graminearum* isolates. In *F. cerealis* isolates, first symptoms on the MR cultivar appeared 10–12 days after single floret inoculation whereas in *F. graminearum* isolates the symptoms appeared on day 5. Similar lower virulence of *F. cerealis* isolates in wheat has been reported in other studies (Sugiura *et al.*, 1994; Desjardins *et al.*, 2004). Miller (1994) reported that the pathogenicity of FHB complex species varied from *F. graminearum* (highest aggressiveness) > *F. culmorum* > *F. avenaceum* > *F. cerealis* (lowest aggressiveness). The regional and annual distribution of FHB complex species is affected by temperature, where *F. culmorum* is more prevalent in cooler climates, followed by *F. cerealis* and *F. graminearum* in warmer climates (Miller, 1994).

Although NIV is not very toxic to plants or wheat, it is more toxic to animals than DON. DON and NIV differ only at the C4 position in the chemical structure: NIV has a hydroxyl group at C4 position whereas DON has a hydrogen. The presence of a hydroxyl group in nivalenol increases the toxicity in animals tenfold compared to the hydrogen in DON (Visconti *et al.*, 1991). NIV contamination is rare in Canada and North America compared to that in Europe and Asia (Desjardins *et al.*, 2004; Schmale *et al.*, 2011). Therefore, discovery of NIV-producing *Fusarium* species in Canada indicates the need for extensive sampling of wheat fields across the country. NIV production is a more ancestral trait and DON-producing isolates have a selective advantage over NIV-producing isolates. But recent reports from the USA on NIV-producing *F. graminearum* and *F. cerealis* isolates and other *Fusarium* isolates with novel traits show the risk of changing profiles of mycotoxins and species in the FHB complex (Starkey *et al.*, 2007; Gale *et al.*, 2011). This study shows the importance of testing for NIV in naturally infected grain samples and the need for extensive research for other *Fusarium* species in the FHB complex that could be a potential threat to the wheat industry in Canada.

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References

Bacon CW, Porter JK, Norred WP, Leslie JE, 1996. Production of fusaric acid by *Fusarium* species. *Applied and Environmental Microbiology* **62**, 403–43.

Bottalico A, Perrone G, 2002. Toxigenic *Fusarium* species and mycotoxins associated with head blight in small grain cereals in Europe. *European Journal of Plant Pathology* **108**, 611–24.

Castanares E, Dinilfo MI, Moreno MV, Beron C, Stenglein SA, 2013. *Fusarium cerealis* associated with barley seeds in Argentina. *Journal of Phytopathology* **161**, 586–9.

Chandler EA, Simpson DR, Thomsett MA, Nicholson P, 2003. Development of PCR assays to *Tri7* and *Tri13* trichothecene biosynthetic genes, and characterisation of chemotypes of *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium cerealis*. *Physiological and Molecular Plant Pathology* **62**, 355–67.

Cumagun CJR, Bowden RL, Jurgenson JE, Leslie JF, Miedaner T, 2004. Genetic mapping of pathogenicity and aggressiveness of *Gibberella zeae* (*Fusarium graminearum*) toward wheat. *Phytopathology* **94**, 520–6.

De Nijs M, Stentor P, Delfgou-van Asch E, Kamphuis H, Rombouts FM, Notermans SHW, 1996. Fungal infection and presence of DON and zearalenone in cereals grown in the Netherlands. *Journal of Food Protection* **59**, 772–7.

Demeke T, Clear RM, Patrick SK, Gaba D, 2005. Species-specific PCR-based assays for the detection of *Fusarium* species and a comparison with the whole seed agar plate method and trichothecene analysis. *International Journal of Food Microbiology* **103**, 271–84.

Desjardins AE, Jarosz AM, Plattner RD, Alexander NJ, Brown DW, Jurgenson JE, 2004. Patterns of trichothecene production, genetic variability, and virulence to wheat of *Fusarium graminearum* from smallholder farms in Nepal. *Journal of Agricultural and Food Chemistry* **52**, 6341–6.

Di Menna ME, Lauren DR, Smith WA, 1991. Effect of incubation temperature on zearalenone production by strains of *Fusarium crookwellense*. *Mycopathologia* **116**, 81–6.

Drummond AJ, Ashton B, Buxton S *et al.*, 2011. GENEIOUS v. 5.4.5. [http://www.geneious.com]. Accessed 10 November 2014.

Fernando WGD, Zhang JK, Dusabenyagasani M, Guo XM, Ahmed H, McCallum B, 2006. Genetic diversity of *Gibberella zeae* isolates from Manitoba. *Plant Disease* **90**, 1337–42.

Gale LR, Harrison SA, Ward TJ *et al.*, 2011. Nivalenol type populations of *Fusarium graminearum* and *F. asiaticum* are prevalent on wheat in southern Louisiana. *Phytopathology* **101**, 124–34.

Garvey GS, McCormick SP, Rayment I, 2008. Structural and functional characterization of the TRI101 trichothecene 3-*O*-acetyltransferase from *Fusarium sporotrichioides* and *Fusarium graminearum*: kinetic insights to combating Fusarium head blight. *Journal of Biological Chemistry* **283**, 1660–9.

Goswami RS, Kistler HC, 2005. Pathogenicity and *in planta* mycotoxin accumulation among members of the *Fusarium graminearum* species complex on wheat and rice. *Phytopathology* **95**, 1397–404.

Guo XW, Fernando WGD, Seow-Brock HY, 2008. Population structure, chemotype diversity, and potential chemotype shifting of *Fusarium graminearum* in wheat fields of Manitoba. *Plant Disease* **92**, 756–62.

Hall TA, 1999. BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–8.

Huelsenbeck JP, Larget B, Miller RE, Ronquist F, 2002. Potential applications and pitfalls of Bayesian inference of phylogeny. *Systematic Biology* **51**, 673–88.

Ichinoe M, Kurata H, Sugiura Y, Ueno Y, 1983. Chemotaxonomy of *Gibberella zeae* with special reference to production of trichothecenes and zearalenone. *Applied and Environmental Microbiology* **46**, 1346–69.

Lauren DR, Minna ME, 1999. Fusaria and Fusarium mycotoxins in leaves and ears of maize plants. 2. A time course study made in the Waikato region, New Zealand, in 1997. *New Zealand Journal of Crop and Horticultural Science* **27**, 215–23.

Lee T, Han YK, Kim KH, Yun SH, Lee YW, 2002. *Tri13* and *Tri7* determine deoxynivalenol- and nivalenol-producing chemotypes of

Gibberella zeae. *Applied and Environmental Microbiology* **68**, 2148–54.

Liddell CM, 2003. Systematics of *Fusarium* species and allies associated with Fusarium head blight. In: Leonard KJ, Bushnell WR, eds. *Fusarium Head Blight of Wheat and Barley*. St Paul, MN, USA: American Phytopathological Society, 35–43.

Logrieco A, Bottalico A, Mule G, Moretti A, Perrone G, 2003. Epidemiology of toxigenic fungi and their associated mycotoxins for some Mediterranean crops. *European Journal of Plant Pathology* **109**, 645–67.

McCormick SP, Alexander NJ, Trapp SE, Hohn TM, 1999. Disruption of *TRI101*, the gene encoding trichothecene 3-*O*-acetyltransferase, from *Fusarium sporotrichioides*. *Applied and Environmental Microbiology* **65**, 5252–6.

McMullen M, Jones R, Gallenberg D, 1997. Scab of wheat and barley: a re-emerging disease of devastating impact. *Plant Disease* **81**, 1340–8.

Miller JD, 1994. Epidemiology of *F. graminearum* diseases of wheat and corn. In: Miller JD, Trenholm HL, eds. *Mycotoxins in Grain: Compounds Other than Aflatoxin*. St Paul, MN, USA: Eagan Press, 19–36.

Miller JD, Greenhalgh R, Wang Y, Lu M, 1991. Trichothecene chemotypes of three *Fusarium* species. *Mycologia* **83**, 121–30.

O'Donnell K, Ward TJ, Geiser DM, Kistler HC, Aoki T, 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genetics and Biology* **41**, 600–23.

Parry DW, Jenkinson P, McLeod L, 1995. Fusarium ear blight scab in small grain cereals – a review. *Plant Pathology* **44**, 207–38.

Posada D, Crandall KA, 1998. ModelTest: testing the model of DNA substitution. *Bioinformatics* **14**, 817–8.

Proctor RH, McCormick SP, Alexander NJ, Desjardins AE, 2009. Evidence that a secondary metabolic biosynthetic gene cluster has grown by gene relocation during evolution of the filamentous fungus *Fusarium*. *Molecular Microbiology* **74**, 1128–42.

Schmale DG, Wood-Jones AK, Cowger C, Bergstrom GC, Arellano C, 2011. Trichothecene genotypes of *Gibberella zeae* from winter wheat fields in the eastern USA. *Plant Pathology* **60**, 909–17.

Šrobárová A, Šliková S, Šudyova V, 2008. Diversity of the *Fusarium* species associated with head and seedling blight on wheat in Slovakia. *Biologia* **63**, 332–7.

Stack R, McMullen M, 1995. A visual scale to estimate severity of Fusarium head blight of wheat. [http://www.ag.ndsu.edu/pubs/plantsci/smgrains/pp1095.pdf]. Accessed 10 November 2014.

Starkey DE, Ward TJ, Aoki T *et al*., 2007. Global molecular surveillance reveals novel Fusarium head blight species and trichothecene toxin diversity. *Fungal Genetics and Biology* **44**, 1191–204.

Sugiura Y, Saito H, Tanaka T, Tanaka T, Ichinoe M, Ueno Y, 1994. *Fusarium crookwellense*, a newly isolated fungus from wheat in Japan: its mycotoxin production and pathogenicity to wheat and barley. *Mycoscience* **35**, 77–82.

Tamura K, Nei M, 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology Evolution* **10**, 512–26.

Tan MK, Simpfendorfer S, Backhouse D, Murray GM, 2004. Occurrence of fusarium head blight (FHB) in southern NSW in 2000: identification of causal fungi and determination of putative chemotype of *Fusarium graminearum* isolates by PCR. *Australian Plant Pathology* **33**, 385–92.

Tittlemier SA, Gaba D, Chan JM, 2013. Monitoring of *Fusarium* trichothecenes in Canadian cereal grain shipments from 2010 to 2012. *Journal of Agricultural and Food Chemistry* **61**, 7412–8.

Ueno Y, 1977. Mode of action of trichothecenes. *Pure and Applied Chemistry* **3**, 1737–45.

Visconti A, Minervini F, Lucivero G, Gambatesa V, 1991. Cytotoxic and immunotoxic effects of *Fusarium* mycotoxins using a rapid colorimetric bioassay. *Mycopathologia* **113**, 181–6.

Ward TJ, Bielawski JP, Kistler HC, Sullivan E, O'Donnell K, 2002. Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. *Proceedings of the National Academy of Sciences, USA* **99**, 9278–83.

Ward TJ, Clear RM, Rooney AP *et al*., 2008. An adaptive evolutionary shift in Fusarium head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America. *Fungal Genetics and Biology* **25**, 473–84.

Yli-Mattila T, Gagkaeva T, 2010. Molecular chemotyping of *Fusarium graminearum*, *F. culmorum* and *F. cerealis* isolates from Finland and Russia. In: Gherbawy Y, Voigt K, eds. *Molecular Identification of Fungi*. Berlin, Germany: Springer Verlag, 159–77.

Zhang H, Chen WQ, Xu J, Xu SJ, Feng J, 2011. First report of *Fusarium cerealis* causing fusarium head blight on barley in China. *Plant Disease* **95**, 774.