Trichothecene and zearalenone production, in culture, by isolates of *Fusarium pseudograminearum* from western Canada


Abstract: One hundred and twenty-seven isolates of *Fusarium pseudograminearum* were obtained from seeds and vegetative parts of cereals and other gramineae grown in western Canada. Culture of the isolates on agar selective for *Fusarium graminearum* allowed a successful differentiation from *F. graminearum*. Sterilized rice (40% moisture content) was inoculated with a single germinated spore of isolates identified as *F. pseudograminearum* and was incubated at 23 °C. After 14 days of incubation, mycelium was taken from each culture, and the DNA was extracted to detect the tricy gene by polymerase chain reaction analysis and confirm the species identity. After 21 days of incubation, mycotoxins were quantified in dried rice. All isolates contained the tricy gene. Deoxynivalenol (DON) was produced in 125 of the 127 isolates, 3-acetyldeoxynivalenol (3-ADON) in 122 isolates, 15-acetyldeoxynivalenol (15-ADON) in 2 isolates, diacetoxyscirpenol (DAS) in 17 isolates, and zearalenone in 100 isolates; both nivalenol (NIV) and fusarenon X (FX) were detected in 1 isolate. There appears to be three chemotypes: DON-3-ADON, DON-15-ADON, and NIV. Neither of the two 15-ADON producers or the NIV producer formed DAS. This is the first time that the production of DAS and FX is associated with *F. pseudograminearum*. None of the isolates produced HT-2 toxin or T-2 toxin at detectable levels.

Key words: *Fusarium pseudograminearum*, wheat, mycotoxins, detection methods, trichothecenes, zearalenone, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, diacetoxyscirpenol, nivalenol, fusarenon X.

Résumé: Cent vingt-sept isolats du *Fusarium pseudograminearum* furent obtenus à partir de graines et d’organes végétatifs de céréales et d’autres graminées cultivées dans l’Ouest canadien. La mise en culture de ces isolats sur gelose sélective pour le *Fusarium graminearum* a permis de les distinguer de ceux du *F. graminearum*. Du riz stérilisé (contenu en humidité, 40%) fut ensemencé avec une seule spore germée des isolats identifiés comme étant le *F. pseudograminearum*, puis fut incubé à 23 °C. Après 14 jours d’incubation, du mycélium a été prélevé à partir de chaque culture pour en extraire l’ADN en vue d’y détecter la présence du gène tricy par analyse de la réaction en chaine de la polymérase et de confirmer l’identité de l’espèce. Après 21 jours d’incubation, les cultures sur riz ont été séchées pour le dosage des mycotoxines. Le gène tricy était présent dans tous les isolats. Le désoxyxivanéol (DON) a été produit par 125 des 127 isolats, le 3-acétyldésoxyxivanéol (3-ADON) par 122 isolats, le 15-acétyldésoxyxivanéol (15-ADON) par 2 isolats, le diacétyloxyxérine (DAS) par 17 isolats et la zéaralénone par 100 isolats; un seul isolat a produit tant le nivaléol (NIV) que la fusarénone X (FX). Il semble ressortir trois chénotypes : le DON-3-ADON, le DON-15-ADON et le NIV. Le DAS n’a pas été retrouvé chez les deux isolats producteurs de 15-ADON ni chez celui producteur du NIV. C’est la première fois qu’on mentionne la production du DAS et de la FX par le *F. pseudograminearum*. Aucun des isolats n’a produit la toxine HT-2 ou la toxine T-2 en quantité décelable.

Mots clés : *Fusarium pseudograminearum*, blé, mycotoxines, méthodes de détection, trichothécènes, zéaralénones, désoxyxivanéol, 3-acétyldésoxyxivanéol, 15-acétyldésoxyxivanéol, diacétyloxyxérine, nivaléol, fusarénone X.
Introduction

In 1977, Francis and Burgess separated *Fusarium graminearum* Schwabe into groups 1 and 2. Group 1 was primarily a crown-rot pathogen and did not form perithecia in culture, whereas group 2 attacked the upper parts of the host and readily formed perithecia. Group 1 is now recognized as a separate species under the name of *F. pseudograminearum* Aoki and O’Donnell, whereas group 2 retains the name of *F. graminearum* (Aoki and O’Donnell 1999). *Fusarium pseudograminearum* is infrequently seedborne, whereas *F. graminearum* commonly infects seed (Burgess et al. 1994; Francis and Burgess 1977; Wiese 1987). Distinguishing these species on the basis of morphology is difficult (Aoki and O’Donnell 1999), but recently, the use of polymerase chain reaction (PCR) (Nicholson et al. 1998; Aoki and O’Donnell 1999) and the development of a selective agar (Poulver et al. 2003) have allowed a reliable separation of *F. graminearum* and *F. pseudograminearum*. The toxigenic potential of *F. pseudograminearum* isolates from Canada has not been assessed, but isolates of this species from Australia have been found to be capable of forming deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), and nivalenol (NIV) (Monds et al. 2005). *Fusarium pseudograminearum* is considered the most important causal agent of *Fusarium* diseases of wheat in Australia (Burgess et al. 2001). Although primarily a pathogen of cereal crowns (Burgess et al. 1994), it can infect the head, causing fusarium head blight (Burgess et al. 1987). The aim of the present study was to assess the capability of Canadian isolates of *F. pseudograminearum*, collected from seed and vegetative cereal parts, to produce toxins in culture.

Materials and methods

Isolation of fungi for inoculation

Between 1998 and 2001, a collection of putatively identified *F. graminearum* isolates collected from *Fusarium*-damaged kernels (FDK) or crop residue from western Canada, but which failed to form perithecia in culture, were stored on SNA (Spizeller Nährstoffarmer agar; Nirenberg 1981) slants at 4 °C. These isolates were screened by transferring mycelium to *F. graminearum* agar (FGA; Quelab Laboratories Inc., Montréal, Que.) (Poulver et al. 2003), a medium selective for *F. graminearum*, and incubating the plates for 7 to 10 days at 30 °C. In 2002 and subsequent years, isolates suspected to be *F. pseudograminearum* on the basis of colony and spore morphology were transferred directly to FGA. *Fusarium graminearum* isolates transferred to FGA medium produce an intense red colour, whereas other *Fusarium* species are typically colourless or nearly so (Fig. 1). Isolates that failed to produce the typical red colour of *F. graminearum* were considered to be *F. pseudograminearum*. The 127 isolates collected were maintained on SNA at 4 °C. Isolates from spring wheat seed were collected between 1998 and 2004, whereas isolates from grass and crop residue were collected from 2001 to 2004. Seventy-one isolates of *F. pseudograminearum* were collected from seed, of which 59 were from FDK. Fifty-six isolates were collected from vegetative plant parts. There were 110 isolates from Alberta, primarily from the southern area of the province, and 17 from Saskatchewan. Because of space constraints, only 40 of the 127 isolates, representing a range of toxin profiles and locations, were deposited at the National Mycological Herbarium in Ottawa, Ontario (Table 1).

Inoculation and growth in solid-state culture

Spores from the cultures growing on SNA were transferred to 0.5 mL of an acid wash solution (two drops of 25% lactic acid in 10 mL of sterile water) for 5 min, then spread onto a potato dextrose agar (PDA) plate and incubated at room temperature for 16 h. For each isolate, a single germinated spore was removed and placed into a 250 mL Erlenmeyer flask containing 50 g of autoclaved long-grain white rice with a moisture content (MC) of 40%. Controls consisted of flasks inoculated with a small block of PDA. Inoculated flasks were placed in sealed plastic bags (12 flasks per bag), with an open beaker of water to maintain humidity, and kept at 23 °C under ambient fluorescent lighting conditions. The bags were opened for a few minutes each week to allow for air exchange. After 14 days of growth, mycelium from each flask was transferred to a fresh SNA slant to preserve the isolate while another portion of mycelium was placed into a 2 mL microcentrifuge tube for DNA extraction and species confirmation by PCR. After 21 days of incubation, the flasks were opened and placed in an oven at 110 °C to dry the rice. The dried rice was removed from the flasks and stored at −15 °C until ground for toxin analysis.

Determination of moisture content

The amount of water required to raise the MC of the 50 g of rice in the flasks to 40% was determined in a two-stage oven moisture assessment (American Association of Cereal Chemists 1981). After the required 25 mL of distilled water was added, the mouths of the flasks were plugged with cotton batting and covered with tin foil. The rice was then al-
Table 1. Sample information and toxin profile for selected isolates, from western Canada, of *Fusarium pseudograminearum* grown on autoclaved rice.

<table>
<thead>
<tr>
<th>DAOMa</th>
<th>Year isolate collected</th>
<th>Townb</th>
<th>Province and districtc</th>
<th>Tissueb</th>
<th>DON</th>
<th>3-ADON</th>
<th>15-ADON</th>
<th>DAS</th>
<th>ZEA</th>
<th>NIV</th>
<th>FX</th>
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<td>FDK</td>
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<td>FDK</td>
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<td>40.1</td>
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*Isolates deposited at the National Mycological Herbarium in Ottawa, Ontario, Canada.

*NA*, not available.

aAlberta; S, Saskatchewan. The numbers or numbers followed by letter(s) represent the crop districts.

bFDK, Fusarium-damaged kernel of wheat.

cND, nondetectable at a detection limit of 0.05 ppm for deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), diacetoxyscirpenol (DAS), nivalenol (NIV), and fusarenon X (FX) and at a detection limit of 0.2 ppm for zearalenone (ZEA).

dAutoclaved rice at 40% moisture content inoculated with an agar block.

Abbreviations: DON, deoxynivalenol; ZEA, zearalenone; FX, fusarenon X.

lowed to imbibe the water overnight prior to being autoclaved and placed into sealed plastic bags to await inoculation. Two flasks were sacrificed to determine the MC of the autoclaved rice prior to inoculation and incubation, while two flasks inoculated only with a small block of PDA were also tested for MC at the end of the day 21 of incubation.
PCR for identification of *F. pseudograminearum* and tri5 gene

DNA extraction and PCR for detection of the trichodiene synthase (tri5) gene and species identification were done according to Demeké et al. (2005). The primer pair FP1-1/FP1-2 (elongation factor-1 gene) used for identifying *F. pseudograminearum*, was that of Aoki and O'Donnell (1999), whereas the tri5-gene-specific PCR primer pair Tox5-1/Tox5-2 was from Niessen and Vogel (1998). Polymerase chain reaction was performed in 96-well plates containing 25 µL of a reaction mixture consisting of: MgCl2, 1.5 mmol/L; KCl, 50 mmol/L; Tris–HCl (pH 8.3), 10 mmol/L; deoxyribonucleoside triphosphates (dNTPs), 0.2 mmol/L of each of four; oligonucleotide primers, 0.4 µmol/L of each; and Taq DNA polymerase (Applied Biosystems, Foster, Calif.), 0.75 U (1 U = 16.67 nkat). For species identification, 1.5 µL of DNA (out of the 200 µL of stock, ∼30 to 50 ng of DNA) was used directly in the 25 µL PCR. DNA amplification was performed in a PTC-200 thermal cycler (MJ Research, Watertown, Mass.), using an initial 3 min denaturation at 95 °C; and then 36 cycles of 30 s at 95 °C, 20 s at 57 °C, and 45 s at 72 °C, followed by a final extension of 5 min at 72 °C. An annealing temperature of 62 °C was used for determination of the presence of the tri5 gene. Amplification products were separated by electrophoresis in 2.0% (w/v) agarose gels stained with ethidium bromide at a concentration of 0.2 µg/mL.

**Assay for trichothecenes**

For mycotoxin analysis, the rice was ground in a hand-held coffee grinder. Sample grinding was carried out in a fume hood. Analyses for DON, NIV, 3-ADON, 15-ADON, fusarenon X (FX), diacetoxyscirpenol (DAS), HT-2 toxin, and T-2 toxin were carried out following a modification of the sample preparation procedure described by Tacke and Casper (1996). A 1 g portion of the ground sample was extracted for 1 h with 20 mL of an acetone:water mixture (84:16, v/v) on a reciprocating shaker. A 9 mL extract was eluted through a 1.5 cm cleanup column containing bonded C18 silica gel and neutral aluminum oxide in a ratio of 1:1 (m/m). Six millilitres of the eluate were evaporated to dryness and derivatized with a trimethylsilylimidazole–trimethylchlorosilane mix. The trimethylsilyl derivaties of the trichothecenes were identified and quantified using a gas chromatograph, Agilent model 6890 (Agilent, Wilmington, Del.), equipped with an autoinjector, mass selective detector (Agilent model 5973N), and a 30 m × 0.25 mm × 0.25 μm DB5-MS column (Agilent). Limit of detection for the trichothecenes was 0.05 ppm.

*Fusarium* trichothecene standards of DON, NIV, 3-ADON, 15-ADON, FX, DAS, HT-2 toxin, and T-2 toxin were purchased from Pierce Chemical Co. (Rockford, Ill.). Mass selective detector (Agilent model 5973N), and a 30 m × 0.25 mm × 0.25 μm DB5-MS column (Agilent). Limit of detection for the trichothecenes was 0.05 ppm.

**Assay for zearalenone**

Determination of ZEA was by liquid chromatography (LC), according to Langseth et al. (1989). The high ZEA levels in the inoculated rice samples required the procedure to be modified. For each isolate, a 12 g sample of ground rice containing 2 g of Celite® was extracted with 10 mL of H3PO4 (0.1 mol/L) and 60 mL of CHCl3. The samples were shaken on a flatbed shaker for 1.5 h and centrifuged at 3000 r/min (1550g) for 10 min at 4 °C. The organic layer was filtered and 2.5 mL of this extract was concentrated to dryness and reconstituted in 10 mL of dichloromethane. This portion of the sample was cleaned up on a silica gel Sep-Pak® cartridge, producing two fractions. The fraction containing ZEA was run on the LC with fluorescence detection. The detection limit for ZEA was 0.2 ppm. The LC system used was a Waters model LCM1 equipped with a Waters model 470 fluorescence detector (Waters, Milford, Mass.). The column was a Waters Symmetry C18 5 μm, 3.9 mm × 150 mm, operated at a flow rate of 0.9 mL/min with a water (pH 3.3 with acetic acid) – acetonitrile gradient. Limit of detection for ZEA was 0.2 ppm.

The ZEA standard was purchased from Sigma Chemical Co.

**Results and discussion**

The initial screening of potential *F. pseudograminearum* isolates based on the absence of perithecia did not exclude all *F. graminearum* isolates, but did reduce the number of isolates that required further screening by FGA and PCR. The results of FGA and species-specific PCR agreed for all isolates, indicating that the FGA medium is capable of distinguishing *F. graminearum* from *F. pseudograminearum*. However, occasionally, isolates of *F. graminearum* that do not produce red pigmentation are found in culture. *Fusarium graminearum* 8ga would not be suitable for those isolates.

The tri5 gene was detected in all isolates, although 1 of the 127 isolates did not produce detectable levels of trichothecenes. In a previous paper (Demeké et al. 2005), the tri5 gene was detected in only 5 of 18 isolates. However, retesting these same isolates demonstrated that they contained the tri5 gene, and all the isolates that were negative for the gene in the previous study produced trichothecenes when grown on rice. The PCR in the previous work may not have been optimized, as there were consistent and robust amplifications of the tri5 gene product in the present work (Fig. 2).

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**Fig. 2.** Amplification of DNA from several isolates of *Fusarium pseudograminearum* from western Canada. Lanes 1–5 amplified with FP1-1/FP1-2 primer set (*F. pseudograminearum* specific, ∼523 base pairs (bp)); lanes 6–11 amplified with Tox5-1/Tox5-2 primer set (∼685 bp). M, low molecular mass DNA ladder (Invitrogen, La Jolla, Calif.). Lanes 1 and 6 are controls (no DNA).
Fig. 3. Range in deoxynivalenol (DON) production by 127 isolates of Fusarium pseudograminearum, from western Canada, grown on autoclaved rice.

Of the 127 isolates, 125 were found to produce detectable levels of DON, 122 produced 3-ADON, 2 produced 15-ADON, 17 produced diacetoxyscirpenol (DAS), 100 produced ZEA, and 1 produced both NIV and FX. Deoxynivalenol levels ranged from nondetectable (ND) to 76.9 ppm; 3-ADON, from ND to 108.7 ppm; 15-ADON, from ND to 19.0 ppm; and DAS, from ND to 1.6 ppm (ND = 0.05 ppm, based on detection limit of the trichothecenes). Zearalenone levels ranged from ND to 267 ppm (ND = 0.2 ppm).

Nivalenol and FX were present at 26.1 and 6.0 ppm, respectively. Average levels of DON, 3-ADON, and ZEA in the 127 samples were 12, 9, and 16 ppm, respectively. Approximately half of the isolates produced less than 10 ppm of DON (Fig. 3). Comparisons with Australian isolates for the amount of toxin produced is difficult as differences in incubation conditions and substrate will alter the level of toxin produced. However, Australian isolates produced the same DON–3-ADON toxin profile as the majority of Canadian isolates (Blaney and Dodman 2002), but in Canada and New Zealand (Monds et al. 2005), there exists also a NIV chemotype. In addition, Canada has a DON–15-ADON chemotype. Neither of the two 15-ADON or the single NIV producer formed DAS. To our knowledge, this is the first time that DAS and FX have been associated with F. pseudograminearum. Western Canadian isolates of Fusarium culmorum (Wm.G. Sm.) Sacc. also formed DON–3-ADON on rice, whereas F. graminearum formed DON–15-ADON (Abramson et al. 2001). Both Canadian and Australian isolates also produced ZEA. Although ZEA was not sought in the previous study of western isolates of F. culmorum and F. graminearum (Abramson et al. 2001), these species are known ZEA producers (Maranas et al. 1984).

The MC of the rice in the controls remained essentially unchanged. Moisture content in the first set of samples was 39.6% prior to incubation and 39.8% afterwards, and in the second set it was 39.7% immediately after autoclaving and 39.6% prior to incubation and 39.8% afterwards, and in the third set it was 39.7% immediately after autoclaving and 39.6% after 3 weeks of incubation. This demonstrates that the method employed retained the target moisture level throughout the 3-week incubation period.

Fusarium pseudograminearum is not a common component of the seed mycoflora in western Canada, but does appear more often on crown and root tissues (Alberta Agricultural Research Institute 2005; Turkington et al. 2005), which is consistent with reports by Burgess et al. (1994), Francis and Burgess (1977), and Wiese (1987). It was also found primarily in the drier areas of the prairies, which is consistent with observations by Blaney and Dodman (2002) in Australia and suggestions by Sung and Cook (1981) that F. pseudograminearum is adapted to dry conditions, as a soilborne pathogen, whereas F. graminearum is adapted to more humid environments, as an airborne pathogen (Dill-Macky 2003). Burgess (1981) also found F. pseudograminearum more frequently in drier regions compared with F. graminearum, which was more abundant in moister environments. Although Burgess et al. (1987) found that F. pseudograminearum caused fusarium head blight on cereals in Australia in 1983, this infrequent situation was attributed to unusually high levels of precipitation in the normally dry area. Burgess et al. (1987) also reported that low levels of trichothecenes were detected on some of the infected grain samples. It is not known what toxins F. pseudograminearum may produce on naturally infected grain in western Canada, but eight isolates inoculated onto wheat in a growth-cabinet trial produced much less DON than did F. graminearum (an average of 43 ppm vs. 208 ppm) (Alberta Agricultural Research Institute 2005; T.K. Turkington unpublished data). The low frequency of the fungus on seed and its presence primarily as a crown-rot pathogen in the drier areas of the prairies will likely limit its impact on grain quality.

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References


