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Mycotoxin producing potential of *Fusarium graminearum* isolates from Uruguayan barley

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Abstract

Twelve isolates of *Fusarium graminearum* were obtained from barley grains collected from different Uruguayan regions (harvest 1993–94). This was the predominant fungal species contaminating the crop due to a particular humid and warm season with cold nights conducive to toxin production The isolates were grown on moist, sterile rice, extracted with aqueous methanol, and examined for mycotoxin production. Zearalenone (ZEA) and the trichothecenes deoxynivalenol (DON), 3- and 15-acetyl-DON (AcDON), nivalenol (NIV), fusarenon-X (FX) and T-2 toxin (T-2) were analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) and confirmed by gas chromatography–mass spectrometry (GC–MS).

Eleven of the 12 strains were DON and/or ZEA producers and 9 were AcDON positive. No NIV, or FX were detected. One strain produced T-2. The predominant acetyl-DON isomer was 15-AcDON. Mass-spectral analysis yielded detectable levels of other mycotoxins, 13-OH-apotrichothecenes, 11-epiapotrichothecenes, culmorin, sambucinol, and isotrichodermol being the most numerous.

From the metabolic profiles it is suggested that Uruguayan *F. graminearum* strains belong to the chemotype IB (DON/15-AcDON). The predominance of this chemotype is in accordance with data from Canada, United States, Mexico and Argentina which have similar climatic conditions that would favor *F. graminearum* growth and mycotoxin production.

Key words: Barley, Deoxynivalenol, *Fusarium graminearum*, Mycotoxins, Trichothecenes, Zearalenone

Introduction

Uruguay's main economic assets come from agriculture, with wheat, corn, barley and rice its most important crops. *Fusarium* disease affects all these grains and is caused mainly by *F. graminearum* [1,2]. Fungal development and mycotoxin formation are dependent on climatic conditions [3–6]. The fungal infection of grains by species of *Fusarium* is a common phenomenon in countries with temperate climates. In the River Plate region the 1993–94 period was particularly humid, warm and with cold nights, conducive to toxin production. *Fusarium* contamination caused severe losses to agriculture and animal productivity. Toxigenic *Fusarium* species are now recognized to be a major agricultural problem with the production of a large number of toxic secondary metabolites. These toxic metabolites include ZEA and the trichothecenes [2,7,8]. The toxigenic potential of monosporic *F. graminearum* strains isolated from barley grains collected from different regions of the country was determined by assaying for zearalenone and trichothecenes (DON, 3- and 15-AcDON, NIV, FX and T-2).

Material and methods

Isolation of Fusaria. Samples ware collected from barley crops, harvest 1993–94 from 4 different regions of Uruguay. The code names indicate the location. One hundred grains from each location were surface disinfected with 1% sodium hypochlorite and 70% ethanol followed by three washes with sterile, distilled water and 5 grains/petri dish placed on dichloran–

chloramphenicol-peptone agar, DCPA [9] for 7 days at 25 °C. Selected *Fusaria* were subsequently subcultured onto potato dextrose agar, PDA [10] incubated for 7 days at 25 °C and on synthetic nutrient agar consisting of KH_2PO_4, 1 g; KNO_3, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; glucose, 0.2 g; saccharose, 0.2 g; agar, 20 g and distilled water to 1 liter, with filter paper for 14 days at 25 °C. Cultures showing micro- and macroconidia in accordance to the *F. graminearum* species concept of Nelson *et al.* [11] were purified by single spore isolation and 12 of these isolates were chosen at random for determination of toxigenic potential.

Culture methods. Fungal cultures were prepared [12] in 250 erlenmeyer flasks containing 50 g of sterile, moist rice (50% moisture). They were inoculated with 2.5 ml of a spore suspension of each selected monosporic *F. graminearum* strain prepared by flooding the slant with 5 ml of sterile distilled water and vigorous agitation. The strains had been grown in PDA for 7 days at 25 °C. Incubation was carried out in the dark for one week at 25–28 °C and 2 weeks at 12 °C. At the end of the incubation period the moldy rice was extracted with 250 ml of methanol–water (45:55, v/v) containing 2.5% NaCl. Hexane (100 ml) was added and agitated for 30 min on a rotary shaker and filtered through Whatman No. 2 paper. The methanol-water phase was extracted 4 times with 25 ml chloroform each time and the combined extracts evaporated on a rotary evaporator at 45 °C. The residue obtained upon removal of the solvents was analyzed for ZEA and trichothecenes by TLC and HPLC.

Chemical analyses. Standards. Standards were from Agriculture Canada, Sigma Chemical Co. and Wako Chemicals. For HPLC they were dissolved in 4 ml of the solvent system to give concentrations of 2 μg ZEA, 1 μg NIV, 2 μg DON, 4 μg FX, 4 μg 3-AcDON and 4 μg 15-AcDON/ml. Injections of 20 μl of combined trichothecene standards and 20 μl of ZEA separately were used. For TLC analysis, standard concentrations were 50 μg ZEA, 100 μg NIV, 20 μg DON, 100 μg FX, 50 μg T-2, 100 μg, 3- and 100 μg 15-AcDON/ml. One, 3, 5 and 10 μl were spotted of each standard.

TLC. Extracts were dissolved in 1000 μl of chloroform and screened for ZEA, DON, 3- and 15-AcDON, NIV, FX and T-2 on precoated 0.20-mm thick silica gel plates without fluorescent indicator (E. Merck) using benzene–acetone (3:2, v/v), chloroform–acetone–isopropanol (8:1:1, v/v) as solvent systems and ethyl acetate–hexane (1:1, v/v) for the 3- and 15-AcDON separation. Five and 20 μl of the sample extracts were spotted in triplicate (250 mg and 1 g concentrations) with both internal and external standards used for positive mycotoxin identification. ZEA was observed at 254 nm and trichothecenes at 365 nm. ZEA was confirmed and Group B trichothecenes were detected by spraying with aluminum chloride (15% in ethanol–water, 15:85, v/v), heating at 120 °C for 2 min and observing blue fluorescence. Group A trichothecenes were sprayed after Group B detection with sulfuric acid (25% in water), heated at 120 °C for 2 min and observed as brownish–green fluorescent spots; ZEA turns orange and Group B trichothecenes disappear.

HPLC. Sample preparation. Equivalent to 50 g culture of each crude fungal extract was dissolved in 2 ml methanol to give 25 g/ml and 50 μl (1.25 g) of each were cleaned up on SPE-C18 columns (Supelco 0.5 g/6 ml). Preconditioning of the column was done with two 6-ml methanol washes followed by two 6-ml distilled water washes each with drainage under gravity. The vials containing the fungal extract were washed with three times 500-μl distilled water and added to the preconditioned columns. Columns were washed with 5.5 ml distilled water which was discarded. Trichothecenes and ZEA were eluted with 6 ml methanol–water (8:2, v/v) into a 50-ml round bottom flask. The eluted fraction was evaporated to dryness on a rotary evaporator at 45 °C, reconstituted by dissolving in 1 ml methanol to give 1.25 g/ml and kept in 4-ml screw cap vials with teflon liners. Before analysis the extracts were adjusted in the mobile phase to a concentration of 0.1 g/ml. Injections were 20 μl of each sample. HPLC was carried out using a Shimadzu LC10-AD pump, Rheodyne 7125 injector and a Waters model 440 UV/Vis detector (229 nm). A 5-μm Ultratechsphere C18 column (250 × 4.6 mm) was used with a flow rate of 1.5 ml of solvent per min. The mobile phase used for Type B trichothecenes was methanol–water (30:70, v/v) and acetonitrile–water (45:55, v/v) for ZEA. Retention times were 3.6 min for NIV,. 5.3 min for DON, 7.6 min for FX, 15.9 min for AcDON and 10.8 min for ZEA.

HPLC was also carried out with a Hewlett Packard 1050 isocratic system with a variable wavelength UV/VIS detector (229 nm) and a 5-μm Hypersil C18 column (100 × 4.6 mm). The same mobile phases and flow rate were used as above.

Mass spectrometry. Toxins were confirmed at the Plant Research Centre, Agriculture and Agri-Food Canada, Ottawa. The fungal extracts prepared as above

Table 1. Mycotoxin production by *F. graminearum* isolates from barley

Isolate	DON (μg/g)[a]	15-AcDON (μg/g)[a]	ZEA (μg/g)[a]	Other mycotoxins identified by GC–MS	Diagnostic m/z
Y2	37.9	Neg.	41.4	7-deoxyDON	280, 250, 249, 231, 203, 189
				8-hydroxyisotrichodermol	266, 251, 224, 218, 177, 123, 109
				15-OH-culmorin	254, 236, 218, 206
Y3	20.3	15.0	80.5	15-OH-culmorin	254, 236, 218, 206
				15-OH-culmorone	252, 234, 219
				3β,13-di-OH-11-epiapo	252, 237, 224
Y4	25.0	11.0	55.0	culmorin	238, 220, 205, 191, 177, 149
				3α,11-oxy-13-OH-apo	250, 235, 219, 161
				isotrichodermol	202, 173, 124
				2,13-di-OH-3α,11-epoxyapo	266, 251, 177, 161
				7-deoxyDON	280, 250, 249, 231, 203, 189
				sambucinol	266, 251, 235, 161, 124
				3α,13-di-OH-11-epiapo	252, 237, 224
				3β,13-di-OH-11-epiapo	252, 237, 224
				15-OH-culmorin	254, 236, 218, 206
Y10	70.2	17.6	88.6	3α,11-oxy-13-OH-apo	250, 235, 219, 161
				3α,13-di-OH-11-epiapo	252, 237, 224
				3β,13-di-OH-11-epiapo	252, 237, 224
				15-OH-culmorin	254, 236, 218, 206
				7-deoxyDON	280, 250, 249, 231, 203, 189
				8-hydroxyisotrichodermol	266, 251, 224, 218, 177, 123, 109
Y17	3.8	6.9	27.9	culmorin	238, 220, 205, 191, 177, 149
				3α,11-oxy-13-OH-apo	250, 235, 219, 161
				3β,13-di-OH-11-epiapo	252, 237, 224
				3α,13-di-OH-11-epiapo	252, 237, 224
				15-OH-culmorin	254, 236, 218, 206
				7-hydroxyisotrichodermol	266, 218, 177, 159, 140, 139, 123, 109
				7-hydroxyisotrichodermin	308, 266, 218, 177, 159, 140, 123, 109
				7-deoxyDON	280, 250, 249, 231, 203, 189
S6	Neg.	Neg.	98.0	culmorin	238, 220, 205, 191, 177, 149
				culmorone	236, 218, 203
				3α,11-oxy-13-OH-apo	250, 235, 219, 161
				isotrichodermol	202, 173, 124
				3β,13-di-OH-11-epiapo	252, 237, 224
				3α,13-di-OH-11-epiapo	252, 237, 224
				12-OH-culmorin	236, 218, 205
				15-OH-culmorin	254, 236, 218, 206
S13	2.7	1.7	9.0	culmorin	238, 220, 205, 191, 177, 149
				culmorone	236, 218, 203
				3α,11-oxy-13-OH-apo	250, 235, 219, 161
				3β,13-di-OH-11-epiapo	252, 237, 224
				3α,13-di-OH-11-epiapo	252, 237, 224
				sambucinol	266, 251, 235, 161, 124
				8-hydroxyisotrichodermol	266, 251, 224, 218, 177, 123, 109
				8-hydroxyisotrichodermin	308, 266, 224, 123, 109
				7-deoxyDON	280, 250, 249, 231, 203, 189

Table 1. Continued

Isolate	DON (μg/g)[a]	15-AcDON (μg/g)[a]	ZEA (μg/g)[a]	Other mycotoxins identified by GC–MS	Diagnostic m/z
S15	70.7	2.7	111	3α,11-oxy-13-OH-apo	250, 235, 219, 161
				isotrichodermol	202, 173, 124
				7-deoxyDON	280, 250, 249, 231, 203, 189
				7-hydroxyisotrichodermol	266, 218, 177, 159, 140, 139, 123, 109
				7-hydroxyisotrichodermin	308, 266, 218, 177, 159, 140, 123, 109
LE2	7.2	10.0	45.8	culmorin	238, 220, 205, 191, 177, 149
				3α,11-oxy-13-OH-apo	250, 235, 219, 161
				isotrichodermol	202, 173, 124
				15-OH-culmorin	254, 236, 218, 206
				3α,13-di-OH-11-epiapo	252, 237, 224
				3β,13-di-OH-11-epiapo	252, 237, 224
				sambucinol	266, 251, 235, 161, 124
LE19	18.0	9.0	60.0	3α,11-oxy-13-OH-apo	250, 235, 219, 161
				isotrichodermol	202, 173, 124
				3β,13-di-OH-11-epiapo	252, 237, 224
				3α,13-di-OH-11-epiapo	252, 237, 224
LE21	30.0	12.0	75.0	culmorin	238, 220, 205, 191, 177, 149
				15-OH-culmorin	254, 236, 218, 206
				3α,13-di-OH-11-epiapo	252, 237, 224
				3β,13-di-OH-11-epiapo	252, 237, 224
DC35P[b]	Neg.	Neg.	Neg.	No relevant toxins	

[a]Values are average of TLC and LC determinations.
[b]T-2 producer by TLC.
None of examined isolates produced NIV or FX by TLC, LC or MS.

Table 2. Deoxynivalenol and zearalenone recoveries

Sample	DON TLC/LC (μg/g)	ZEA TLC/LC (μg/g)	Recoveries (% of added) TLC/LC	Average recoveries (% of added) TLC/LC
Isolate DC35P	100/99.2	100/99.2		
spiked with	98/97	–	98/97	97.6/96.1
100 μg/g DON	95/92		95/92	
Isolate DC35P		50/37.5	100/75	
spiked with	–	49/30.0	98/60	97.3/71
50 μg/g ZEA		47/38.8	94/78	

were taken to dryness under nitrogen, redissolved in ethyl acetate and analyzed using a Finnigan model 4500 gas chromatography/mass spectrometer system [13] with temperature program 100–280 °C at 15 °C/min. Mass spectra ware determined in the electron impact mode and compared with the mycotoxin data base library of the Plant Research Centre, Agriculture and Agri-Food Canada.

Results and discussion

Mycotoxins detected in the *F. graminearum* extracts are reported in Table 1. All DON, AcDON and ZEA TLC and HPLC findings were confirmed by GC–MS. Diagnostic m/z values observed were 296, 278, 265 and 248 for DON; 278, 265, 249, 231 and 203 for 15-AcDON; and 318, 300, 249, 231, 204 and 188 for ZEA, although weaker ions were missing in some cases for DON and 15-AcDON. DON and/or ZEA were consistently produced by 11 of the 12 isolates. AcDON was produced by 9 out of 12 strains tested. The major isomer was 15-AcDON. AcDON separation was achieved by TLC and identity confirmed by GC-MS. The 3- and 15-AcDON were not separated by the HPLC method used. One strain (S6) produced only high yields of ZEA as the main mycotoxin. Isolate Y10 produced high levels of all three toxins, with yields of DON, AcDON and ZEA of 70.2, 17.6 and 88.6 μg/g respectively. Isolate S15 also produced high concentrations while other isolates can be classified as moderate (e.g. Y3, Y4, LE21) and low (e.g. S13, Y17) producers.

Concentrations of major toxins varied. ZEA was produced by 91.7% of the strains (11/12) at an average concentration of 62.9 μg/g (9–111), 83.3% (10/12) produced an average of 28.6 μg/g (2.7–70.7) of DON and 75% (9/12) produced an average of 9.5 μg/g (1.7–17.6) of 15-AcDON. DC35P was unique in that it produced unconfirmed T-2 by TLC but none of the other mycotoxins looked for were positively identified nor did it produce any relevant toxin by HPLC or GC-MS. No NIV or FX were detected in any of the 12 extracts.

Mass-spectral data revealed also a number of minor secondary metabolites [13,14] including derivatives of culmorin, sambucinol, isotrichodermin and isotrichodermol and of apotrichothecene (apo): 13-OH-apo and the epimers (3α-OH and 3β-OH) of 13-hydroxy-11-epiapo as the most commonly present (Table 1). Identifications were based on GC retention time and matching of mass spectra.

Control recoveries were determined by TLC for DON and ZEA (Table 2) with 97.6% and 97.3% as the average recoveries from control cultures spiked at 100 and 50 μg/g, respectively. Recoveries for DON and ZEA ware also measured by HPLC with 96.1% and 71% average recoveries respectively.

The results of toxin production by the isolates tested suggest that there is a large potential for trichothecene, especially DON, and ZEA production in Uruguayan fields. The metabolic profiles of the *F. graminearum* isolates analyzed were similar, having chemotype IB (DON, 15-AcDON) [15] in common.

Conclusions

This study indicates that *F. graminearum* strains isolated from barley are capable of producing a variety of toxins, constituting a potential human and animal health hazard. Barley is an important substrate for feed and the main raw material for malting and beer production, both processes which may not reduce mycotoxin levels significantly [16,17].

The results of this limited survey also indicate that our isolates would belong to the chemotype IB which is in agreement with reports from Canada, the United States, Mexico and Argentina [15,18–21] where this is the dominant chemotype of the species.

The data contribute to furthering our knowledge of chemotypes and geographic distribution and support the premise that there is a regional relationship between the origin of *F. graminearum* and the production of 3- or 15-AcDON as the major isomer. A greater percentage of the isolates producing 15-AcDON are found in North and South America analogous to DON and not NIV production in both continents [22,23].

Further studies involving a greater number of isolates from grain samples are currently under way.

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References

1. Smalley EB, Strong FM. Toxic trichothecenes. In: Purchase IFH (ed), Mycotoxins. Amsterdam: Elsevier, 1974; 199–228.
2. Ueno Y. (ed.), Trichothecenes: Chemical, biological and toxicological aspects. Tokyo, Japan: Kodansha Ltd, 1983; 135–145.

3. Martin RA. Seasonal variability of *Fusarium* species associated with cereal heads. Abstract 2-40, 5th Intl. Congress Plant Pathology, Kyoto, Japan, August 20–27, 1988.
4. Sutton JC. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. Can J Plant Pathol 1982; 4: 195–209.
5. Sutton JC, Baliko W, Funnell HS. Relation of weather variables to the incidence of zearalenone in corn in southern Ontario. Can J Plant Sci 1980; 60: 149–155.
6. Naik DM, Busch LV, Barron GL. Influence of temperature on the strain of *Fusarium graminearum* Schwabe in zearalenone production. Can J Plant Sci 1978; 58: 1095–1097.
7. Foster BC, Trenholm HL, Friend DW, Thompson BK, Hartin KE, Evaluation of different sources of deoxynivalenol (vomitoxin) fed to swine. Can J Anim Sci 1986; 66: 1149–1154.
8. Ichinoe M, Kurata H, Sugiura Y, Ueno Y. Chemotaxonomy of *Gibberella zeae* with special reference to production of trichothecenes and zearalenone. Appl Environ Microbiol 1983; 46: 1364–1369.
9. Andrews S, Pitt JI. Selective medium for isolation of *Fusarium* species and dematiaceous hyphomycetes from cereals. Appl Environ Microbiol 1986; 51: 1235–1238.
10. Booth C. The genus *Fusarium*. Commonwealth Mycological Institute, Kew, England, 1971.
11. Nelson PE, Toussoun TA, Marasas WFO. *Fusarium* species: an illustrated manual for identification. University Park, PA: Pennsylvania State University Press, 1983.
12. Bottalico A, Lerario P, Visconti A. Production of mycotoxins (zearalenone, trichothecenes and moniliformin) by *Fusarium* species in Italy. Microbiol Alim Nutr 1983; 1: 133–142.
13. Greenhalgh R, Fielder DA, Morrison LA, Charland JP, Blackwell BA, Savard ME, ApSimon JW. Secondary metabolites of *Fusarium* species: apotrichothecene derivatives. J Agric Food Chem 1989; 37: 699–705.
14. Greenhalgh R, Meier RM, Blackwell BA, Miller JD, Taylor A, ApSimon JW. Minor metabolites of *Fusarium roseum* (ATCC 28114). 2. J Agric Food Chem 1986; 34: 115–118.
15. Miller JD, Greenhalgh R, Wang YZ, Lu M. Trichothecene chemotypes of three *Fusarium* species. Mycologia 1991; 83: 121–130.
16. Scott PM, Kanhere SR, Daley EF, Farber JM. Fermentation of wort containing deoxynivalenol and zearalenone. Mycotoxin Res 1992; 8: 58–66.
17. Niessen L, Donhauser S. Fate of deoxynivalenol in the process of brewing and its prevalence in commercial beer. In: Scudamore K (ed), Occurrence and significance of mycotoxins. Central Science Lab., Slough, Berks, England, 1993; 203–207.
18. Greenhalgh R, Blackwell BA, Pare JRJ, Miller JD, Levandier D, Meier RM, Taylor A, ApSimon JW. Isolation and characterization by mass spectrometry and NMR spectroscopy of secondary metabolites of some *Fusarium* species. In: Steyn PS and Vleggaar R (eds), Mycotoxins and Phycotoxins. Amsterdam: Elsevier, 1986, 137–151.
19. Miller JD, Taylor A, Greenhalgh R. Production of deoxynivalenol and related compounds in liquid culture by *Fusarium graminearum*. Can J Microbiol 1983; 29: 1171–1178.
20. Banchero EP, Miguel MS, Godoy HM, Di Giulio AM, Dobois MED, Souto G, Gomez M, Resnik FZ, Torres ME. Evaluación de la presencia de *Fusarium* y de micotoxinas en muestras de trigo de la campaña 1985/86 y su relación con la calidad comercial. Public Gerencia Técnica, Junta Nacional de Granos, Argentina, 1987.
21. Lori GA, Carranza MR, Violante A, Rizzo I, Alippi H, Varsavsky E. Presencia de las especies del género *Fusarium* en trigo: quimiotaxonomía y capacidad toxicogénica de las cepas aisladas. Public II Congreso Nacional de trigo, Pergamino, Argentina, 1990.
22. Mirocha CJ, Abbas HK, Windels CE, Xie W. Variation in deoxynivalenol, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, and zearalenone production by *Fusarium graminearum* isolates. Appl Environ Microbiol 1989; 55: 1315–1316.
23. Faifer GC, De Miguel MS, Godoy H. Patterns of mycotoxin production by *Fusarium graminearum* isolated from Argentine wheat. Mycopathologia 1990; 109: 165–170.

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