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Mycotoxin profile of *Fusarium armeniacum* isolated from natural grasses intended for cattle feed

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RESEARCH ARTICLE

Abstract

Fusarium armeniacum has been found as a saprophyte on natural grasses devoted to cattle feed in Argentina. This species has been reported as highly toxigenic due to the production of trichothecenes type A, but the information available about its toxigenic profile is incomplete. Thus, the aim of the present study was to determine the toxigenic ability of 50 *F. armeniacum* isolates recovered from natural grasses using a multitoxin method based on LC-MS/MS. In addition, morphological identification of 15 selected isolates was confirmed by sequencing the translation elongation factor 1α. Out of the 327 metabolites analysed, only 10 were detected: T-2 toxin (T-2), T-2 triol, T-2 tetraol, HT-2 toxin (HT-2), diacetoxyscirpenol (DAS), monoacetoxyscirpenol (MAS), neosolaniol (NEO), aurofusarin (AUF), beauvericin (BEA) and zearalenone (ZEA). The most common group of mycotoxins produced by the isolates on rice under laboratory conditions was trichothecenes type A, and some minor *Fusarium* mycotoxins, such as BEA and AUF. Some isolates were also able to produce ZEA. Among the trichothecene type A, HT-2, T-2, NEO were clearly synthesised at the highest levels and frequency, followed by DAS and MAS. HT-2, T-2, NEO and DAS production was detected in 48 (96%), 47 (94%), 47 (94%) and 38 (76%) isolates, respectively. The ability of *F. armeniacum*, to produce ZEA and AUF has been demonstrated here for the first time. Given the new information provided about the toxigenic profile of this species, commonly associated with natural grasses in Argentina, the threat to animal health posed by this fungus should not be underestimated.

Keywords: *Fusarium,* cattle feed, mycotoxins, trichothecenes type A, zearalenone

1. Introduction

Meat production in Argentina is based on the exploitation of natural sources with traditional extensive grazing. Animals grazing in rangelands, pastures, and grasslands with little or no integration of crops involved are a common practice in Argentina. A previous pilot survey was undertaken to ascertain the presence of multiple mycotoxins (by LC-MS/MS) in 40 samples of natural grasses (*Poaceae*) in two beef cattle farms located in the wetlands of Chaco province in Argentina (Ramirez *et al.*, 2012). The analysis revealed the presence of zearalenone (ZEA) and partial co-occurrence of α and β-zearalenol. T-2 toxin (T-2) and HT-2 toxin (HT-2) were found in high frequency as well. Other fungal metabolites that were found to be prevalent were beauvericin (BEA), equisetin and aurofusarin (AUF), metabolites produced by *Alternaria* spp., sterigmatocystin and its precursors, and anthrachinone derivatives such as emodin, chrysophanol and skyrin. Mycological analysis of the natural grass samples revealed that 100% of the samples were contaminated with *Fusarium* being *Fusarium armeniacum* the most common species found (Nichea, unpublished results). The result is not surprising because representatives of the genus *Fusarium* occur as pathogens, endophytes or saprobes in a wide range of plants and soil worldwide (Leslie and Summerell, 2006; Summerell *et al.*,

2010). Recently surveys have also suggested that *Fusarium* species are commonly found as endophytes of the *Poaceae* in grassland ecosystems in the USA, Australia and Hungary (Leslie *et al.*, 2004; Phan 2006; Sanchez Marquez *et al.*, 2008; Szecsi *et al.*, 2013).

F. armeniacum was initially referred as *F. acuminatum* and then, due to similarities in macroconidial morphology, it was described as a subspecies of *F. acuminatum* (Burgess *et al.*, 1993). However, subsequent studies, e.g. Altomare *et al.* (1997), Benyon and Burgess (1997) and Benyon *et al.* (2000), showed that this taxon was a distinct species and it was raised to species rank by Burgess and Summerell (2000). Based on molecular chromosomal karyotypes, *F. armeniacum* is more closely related to *Fusarium sporotrichioides* than to *F. acuminatum* (Nagy and Hornok, 1994). *F. acuminatum* and *F. armeniacum* can be differentiated on the basis of pigmentation, speed of production of chlamydospores and growth rates. *F. armeniacum* has been found in maize in Minnesota (Kommedahl *et al.*, 1979) and was later recovered in Australia (Burges *et al.*, 1988) and South Africa (Marasas *et al.*, 1988; Rabie *et al.*, 1986). *F. armeniacum* appears to be a saprophyte, until recently, when it was reported as a pathogen on soybean in the United States (Ellis *et al.*, 2012).

This species is highly toxigenic in a chick assay and can produce trichothecenes (Wing *et al.*, 1993, 1994). Strains of *F. armeniacum* contain the *TRI*5 gene which is the first unique step in trichothecene biosynthesis, but *F. acuminatum* strains do not (Fekete *et al.*, 1997). Thus, earlier reports of the production of high levels of trichothecenes (specifically T-2, HT-2 and neosolaniol (NEO)) by *F. acuminatum*, e.g. Rabie *et al.* (1986) and Logrieco *et al.* (1992), are probably best attributed to *F. armeniacum*. The latter produces T-2 and related trichothecenes, and also produces BEA and fusarin C, but according to Desjardins (2006) the mycotoxin profile of this species is still incomplete.

Trichothecenes are agriculturally important mycotoxins that present a potential threat to animal health throughout the world. Trichothecenes are potent inhibitors of eukaryotic protein synthesis, including DNA, RNA synthesis, inhibition of mitochondrial function, effects on cell division and membrane effects (Desjardins, 2006). All trichothecenes have a tricyclic skeleton structure with an epoxide group, but they are divided into two structurally distinct groups based on the absence (type A trichothecenes) and the presence (type B trichothecenes) of a keto group at carbon atom 8 (C-8) of the skeleton. Examples of type A trichothecenes include T-2, HT-2, diacetoxyscirpenol (DAS) and NEO. Clinical signs of trichothecene infection in animals include feed refusal and vomiting, growth retardation, reproductive disorders, blood disorders, dermatitis, oral lesions and depression of the immune response (Li *et al.*, 2011). BEA belongs to the cyclic hexadepsipeptide mycotoxins synthesised by numerous fungi that are considered as emerging *Fusarium* toxins. The extent of human, animal and plant exposure to this mycotoxin has not been well established (Stępień and Waśkiewicz, 2013).

The identification of *Fusarium* species traditionally relies on the detection of both morphological and physiological features. However, discrimination among similar species is often difficult; translation elongation factor 1α gene has been used as a single-locus identification tool and it is a suitable genetic marker for discriminating among *Fusarium* species (Geiser *et al.*, 2004). Because different *Fusarium* spp. possess variable toxigenic potential, accurate identification is necessary.

In the light of the prevalence of *F. armeniacum* in natural grasses intended for cattle feed in Argentina, it is relevant to characterise its toxigenic ability in order to evaluate the toxicological risk of the presence of this species. Thus, the aim of the present study was to determine the toxigenic ability of fifty *F. armeniacum* isolates recovered from natural grasses.

2. Materials and methods

Fungal isolates

The study included 50 isolates of *F. armeniacum.* Single-spored cultures from colonies initially identified as *Fusarium* spp. were transferred to carnation leaf agar (CLA) and potato dextrose agar (PDA) and were further identified based on morphology (Leslie and Summerell, 2006). These were isolated from natural grasses obtained from two beef cattle farms located in Chaco province in Argentina. The sampling was done during winter, and therefore was not possible to identify the grasses (mainly *Poaceae*) up to species level due to the absence of inflorescence. The isolates are deposited at the Department of Microbiology and Immunology, Universidad Nacional de Rio Cuarto culture collection. Cultures are maintained in 15% glycerol at -80 °C.

DNA extraction and PCR amplification of TEF-1 α

Fifteen strains, selected to be representative of the different mycotoxins profiles, were grown in complete medium (Leslie and Summerell, 2006) and incubated on an orbital shaker (150 rpm) for at least three days at 25±1 °C. The resulting mycelia were harvested by filtration through non-gauze milk filters (Ken AG, Ashland, OH, USA). Excess water was removed by blotting mycelia between clean paper towels, and dried mycelia were stored frozen at -20 °C. The DNA extraction was performed with a cetyl-trimethylammnonium bromide method (Leslie and Summerell, 2006).

Analyses of the partial sequence of the translation elongation factor-1α gene (*TEF-1α*), were made following PCR amplification with the primers described by O'Donnell *et al.* (1998). The PCR experiments were conducted with 10-20 ng of fungal DNA in a total volume of 20 µl of 1× reaction buffer containing 1.5 mM $MgCl_2$, 0.5 U Taq DNA polymerase (Promega, Madison, WI, USA), 0.2 mM dNTPs and 0.5 µM of each TEF primer (EF1 5´-ATGGGTAAGGAGGACAAGAC-3´, EF2 5´-GGAAGTACCAGTGATCATGTT-3´). A negative control, containing all reagents and primers but no fungal DNA, was included in every set of reactions. PCR was conducted in a PTC-2000 Thermal Cycler (MJ Research Inc., Watertown, MA, USA) and the conditions were: 94 °C for 1 min, then 31 cycles at 94 °C for 30 sec, 56 °C for 45 sec and 72 °C for 1 min, followed by 72 °C for 5 min and at 4 °C. PCR products were separated by electrophoresis through 2% (w/w) agarose gels to confirm that a ~700 bp fragment was amplified for TEF-1*α*. Fragments were purified by filtration through DNA Wizard Clean-Up Kit (Promega). Sequencing of both strands was performed with an ABI 3130XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and each sequence was then aligned with ClustalW (Thompsom *et al.*, 1994), as implemented in the program BioEdit version 7.0.9.0 (Hall, 1999). A BLAST search for similarities was performed with the sequences obtained.

Mycotoxin production

F. armeniacum isolates were cultured in Erlenmeyer flasks (250 ml) containing 25 g of long grain rice. Ten ml of distilled water was added before autoclaving for 30 min at 121 °C. The procedure was repeated twice. Each flask was inoculated with a 3 mm diameter agar disk taken from the margin of a colony grown on synthetic nutrient agar (Leslie and Summerell, 2006) at 25 °C for seven days. Flasks were shaken by hand once a day for 1 week. These cultures were incubated for 28 days at 25 °C in dark. At the end of the incubation period the contents of the flask were dried at 50 °C for 24 h and then stored at -20 °C until analysed for mycotoxins.

Mycotoxin analysis

In brief, 5 g of each inoculated rice sample were extracted for 90 min with 20 ml of acetonitrile:water:acetic acid (79:20:1, v/v/v) on a rotary shaker (GFL 3017, GFL, Burgwedel, Germany). Extraction, dilution, and analysis were performed as described by Sulyok *et al.* (2007). The raw extracts were diluted in the extraction solvent (ratio 1:10), further diluted (ratio 1:2) with acetonitrile:water:acetic acid (20:79:1, v/v/v) and injected into the LC-MS/MS instrument. Chromatographic separation was performed at 25 °C on a Gemini® C_{18}-column, 150×4.6 mm i.d., 5 µm particle size, equipped with a C_{18} 4×3 mm i.d. security (all from Phenomenex, Torrance, CA, USA) and coupled to an 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Two mixtures containing 5 mM ammonium acetate composed of methanol:water:acetic acid (eluent A: 10:89:1, v/v/v; and eluent B: 97:2:1, v/v/v;) were used as mobile phases in a gradient elution mode. After an initial time of 2 min at 100% eluent A, the proportion of eluent B was increased linearly to 50% within 2-5 min and 100% within 5-14 min, followed by a hold-time of 4 min at 100% eluent B and 2.5 min column re-equilibration at 100% of eluent A pumped at a flow rate was 1 ml/min.

Detection and quantification of the mycotoxins were performed with a QTrap 5500 MS/MS system (Applied Biosystems) with a turbo ion spray electrospray ionization (ESI) source. Electrospray ionization-MS/MS was performed in a scheduled multiple reaction monitoring (sMRM) mode, both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. All samples were analysed using Analyst® software version 1.5.2 (AB Sciex, Foster City, CA, USA).

ESI-source settings: source temperature 550 °C, curtain gas 30 psi (206.8 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 80 psi (551.6 kPa of nitrogen), ion source gas 2 (drying gas) 80 psi (551.6 kPa of nitrogen), ion-spray voltage -4,500 V and +5,500 V, respectively, collision gas (nitrogen) medium. Optimised analyte-dependent MS/MS parameters are given elsewhere (Malachová *et al.,* 2014). Unambiguous identification of the analytes was obtained by the acquisition of two MS/MS transitions per analyte and comparison of the related intensity ratio and the LC retention time to the related values of an authentic standard.

3. Results and discussion

Fifty isolates were morphologically identified as *F. armeniacum*. Colonies on PDA produced white aerial mycelium, red to apricot pigmentation in agar, and bright orange sporodochia in the centre of the culture. On CLA, macroconidia in orange sporodochia on carnation leaves and chlamydospores were formed abundantly. Further, 15 isolates were selected based on the different mycotoxin profile obtained (Table 1) in order to confirm their morphological identification. Species identity for the 15 selected isolates was confirmed by elongation factor gene (EF1-α) sequencing using EF1 and EF2 primers. BLASTn analysis with the FUSARIUM-ID and NCBI database revealed 99 to 99.24 % sequence identity to *F. armeniacum* (NRRL 29133, GenBank Accession No. HM744659). Thus, both morphological and molecular criteria supported identification of the isolates as *F. armeniacum*. To the best of our knowledge, this is the first report with confirmed molecular identification of *F. armeniacum* isolates from natural grasses.

Table 1. Mycotoxin production profiles of *Fusarium armeniacum* isolates on rice culture.[1,2]

Isolate	T-2 (mg/kg)	T-2TE (mg/kg)	T-2TR (mg/kg)	HT-2 (mg/kg)	DAS (mg/kg)	MAS (mg/kg)	NEO (mg/kg)	AUF (mg/kg)	BEA (mg/kg)	ZEA (µg/kg)
*A4 20	16.51	34.80	3.18	7.38	0.04	0.02	4.68	118.56	0.85	ND
A4 17	11.03	ND	1.72	3.50	ND	ND	1.75	41.97	0.48	ND
A4 6	17.98	3.63	ND	5.54	0.08	ND	8.46	5.18	0.36	ND
A9 5	464.40	972.80	36.10	73.13	1.77	0.43	541.60	1,796.80	1.63	ND
A8 9	91.20	124.00	9.87	11.66	0.41	ND	229.52	1,208.80	0.57	ND
*A7 26	1.22	5.48	ND	0.12	ND	ND	9.88	3.49	0.91	ND
A8 25	191.28	314.16	14.35	24.64	0.79	0.11	382.88	1,264.00	1.04	ND
*A1 3	ND	ND	1.09	3.41	ND	ND	ND	ND	0.80	ND
A2 22	ND	ND	ND	0.51	ND	ND	ND	ND	0.50	ND
A5 19	28.91	ND	3.14	6.22	0.04	ND	6.87	1,034.40	1.20	ND
A4 11	101.28	ND	11.35	14.79	0.08	ND	27.02	1,192.80	0.46	ND
A6 3	23.10	92.48	4.28	9.77	ND	ND	2.56	286.48	0.50	ND
A10 12	57.42	314.40	3.72	4.22	0.68	ND	295.92	976.00	1.29	ND
A5 17	25.55	189.92	3.80	5.54	ND	ND	3.14	977.60	0.84	ND
A3 8	ND	ND	5.11	11.46	ND	ND	ND	ND	1.61	ND
*A7 24	24.25	499.60	9.36	50.86	0.17	ND	52.24	1,105.60	0.78	ND
*A6 26	115.20	81.28	10.52	16.88	0.15	ND	48.83	143.92	0.08	ND
A6 8	15.30	25.43	2.41	6.20	ND	ND	2.61	20.10	0.43	ND
A10 10	53.06	13.21	5.54	9.63	0.22	ND	38.68	6.64	7.39	ND
A2 1	134.88	310.88	8.54	18.26	1.14	0.27	419.84	1,095.20	0.27	ND
*A7 13	15.05	ND	0.57	2.66	0.12	ND	50.24	247.84	2.22	ND
A5 21	14.11	42.62	1.87	4.84	ND	ND	3.43	159.36	0.51	ND
A1 12	34.98	ND	3.86	30.03	0.99	0.50	8.10	67.08	0.84	ND
*A6 19	39.18	177.92	6.22	10.91	0.06	0.06	14.36	442.08	0.19	ND
*A1 20	31.82	ND	1.26	4.63	0.24	0.04	49.78	106.08	0.19	ND
*A2 11	90.56	ND	4.83	11.03	0.53	0.17	282.48	829.60	3.64	ND
A5 20	23.02	146.08	2.59	6.10	ND	ND	2.69	375.20	0.81	ND
A9 26	47.25	ND	2.43	3.52	0.41	ND	143.92	490.32	11.34	ND
A2 28	48.05	ND	2.10	3.68	0.42	ND	148.24	479.68	11.29	ND
A9 30	292.72	ND	14.09	31.18	0.89	ND	307.76	950.40	0.95	ND
*A7 27	329.12	98.08	5.30	31.38	1.90	0.32	112.80	65.94	1.64	ND
A10 5	77.73	164.32	22.06	ND	0.55	0.49	239.84	272.48	0.35	ND
A3 22	19.18	ND	1.95	2.04	0.36	ND	200.16	509.60	29.69	ND
*A9 12	120.40	59.34	3.27	10.26	0.30	0.06	58.34	345.52	0.03	ND
*A1 14	70.07	54.86	7.34	29.79	0.28	ND	160.16	110.00	15.02	ND
A6 2	23.88	ND	1.31	3.24	0.85	ND	54.15	9.17	25.04	ND
A8 22	23.75	ND	1.92	7.94	0.11	ND	5.71	16.53	0.46	ND
B2 7	250.80	ND	5.24	16.10	2.36	0.34	344.08	520.00	0.69	13.95
*B1 4	119.28	ND	54.62	146.80	0.10	ND	184.88	220.00	25.76	24.21
A8 6	547.44	ND	15.18	56.13	3.45	0.74	574.80	1,276.00	0.24	46.30
B3 20	67.34	ND	2.66	3.10	1.17	ND	231.04	862.40	0.58	167.92
*A1 1	168.80	ND	14.26	96.32	0.91	0.45	173.68	163.60	ND	ND
*X3 15	192.16	ND	1.00	10.10	1.73	0.21	194.24	322.24	0.85	ND
X1 9	142.24	163.68	11.18	102.48	0.78	1.06	149.44	360.00	14.26	ND
B3 16	0.58	2.79	ND	0.49	ND	ND	14.74	20.19	20.82	1,173.60
B3 13	346.96	ND	9.66	21.81	2.42	0.27	374.64	1,035.20	0.64	210.64
B2 19	39.91	ND	3.65	4.73	0.56	0.13	184.88	318.24	1.95	51.46
A9 23	0.61	ND	ND	ND	ND	ND	1.20	3.09	ND	78.74
X2 19	89.60	123.60	7.83	42.79	0.67	0.74	94.32	34.86	0.99	80.48
B1 15	115.92	150.80	2.44	8.38	0.97	0.12	179.20	255.52	19.94	4.03

[1] AUF = aurofusarin; BEA = beauvericin; DAS = diacetoxyscirpenol; HT-2 = HT-2 toxin; MAS = monoacetoxyscirpenol; NEO = neosolaniol; T-2 = T-2 toxin; T-2TE = T-2 tetraol; T-2TR = T-2 triol; ZEA = zearalenone.

[2] ND = not detected, i.e. below limit of detection. Strains labelled with an asterisk (*) were analysed by the partial sequence of the translation elongation factor gene.

Three hundred and twenty seven metabolites were analysed by LC-MS/MS for all the studied isolates. Only two isolates were able to co-produce the following 10 mycotoxins: T-2, T-2 triol (T-2TR), T-2 tetraol (T-2TE), HT-2, DAS, monoacetoxyscirpenol (MAS), NEO, AUF, BEA and ZEA (Table 1). The more frequent profiles of toxin production found were the co-production of T-2, T-2TR, HT-2, DAS, MAS, AUF and BEA by 9 isolates followed by T-2, T-2TR, T-2TE, HT-2, DAS, MAS, NEO, AUF and BEA by 8 isolates. The most common mycotoxin group produced was trichothecene type A, although some minor *Fusarium* mycotoxins, such as BEA and AUF, were also produced. Some isolates were also able to produce ZEA.

Among the trichothecene type A, HT-2, T-2, NEO were clearly synthesised at the highest levels and frequency, followed by DAS and MAS. HT-2, T-2, NEO and DAS production was detected in 48 (96%), 47 (94%), 47 (94%) and 38 (76%), isolates respectively. The levels ranged from 0.58 to 547 mg/kg (mean concentration positive isolates = 101.17 mg/kg) for T-2, 1.20 to 542 mg/kg (mean concentration positive isolates = 141 mg/kg) for NEO, 0.12 to 147 mg/kg (mean concentration positive isolates = 20.55 mg/kg) for HT-2 and 0.04 to 3.45 mg/kg (mean concentration positive isolates = 0.76 mg/kg) for DAS. Other trichotheces type A produced in low frequency was MAS (20 isolates). The related T-2 derivates, T-2TR and T-2TE, have also been detected. T-2TR was produced by 45 isolates (90%) in levels ranging from 1.0 to 54.62 mg/kg, whereas T-2TE was produced by 23 isolates (46%) at levels ranging from 2.79 to 973 mg/kg.

BEA was produced by 48 isolates (96%) at levels ranging from 0.03 to 29.7 mg/ kg with a mean concentration of positive isolates of 4.44 mg/kg. Almost all isolates (47 out of 50) were able to produce AUF at levels ranging from 3.09 to 1,797 mg/kg with a mean concentration of positive isolates of 471 mg/kg. ZEA was produced in low frequency (10 isolates, 20%) at concentrations ranging from 4.03 to 1,173.60 µg/kg.

The toxin pattern detected in the *F. armeniacum* cultures was partially in agreement with earlier studies conducted on the same species isolated from different regions of the world. *F. armeniacum* isolated during the present study were able to produce T-2, T-2TR, T-2TE, HT-2, DAS, MAS, NEO, AUF, BEA and ZEA in low frequency. It has been previously reported, that *F. armeniacum* isolates can produce trichothecene mycotoxins such as T-2, HT-2, DAS and NEO (Abbas *et al.*, 1989; Logrieco *et al.*, 1992; Moss and Thrane 2004; Rabie *et al.*, 1986). According to Desjardins (2006) *F. armeniacum* produces T-2 and related trichothecenes, and also produce BEA and fusarin C, however mycotoxin profiles of this species was incomplete. Zitomer (2006) showed that 3 isolates of *F. armeniacum* have the ability to produce trichothecenes type A and B but were unable to produce ZEA. Isolate NRRL 6227 showed production of NIV, DON, NEO, HT-2, and T-2. Isolate NRRL 29133 showed production of NIV, DON, FUS-X, NEO, and DAS. Isolate NRRL 31970 showed production of NIV, DON, ADON, NEO, DAS, and T-2. The current isolates were unable to produce type B trichothecenes and fusarin C.

In the present study, nearly all the isolates of *F. armeniacum* produced AUF, a typical red pigment characteristic of many *Fusarium* species. The ZEA production was scattered among the isolates under study. To our knowledge the production of these compounds has not been previously reported for this *Fusarium* species and should be added to the scarce available information for this species.

Furthermore, it has to be stressed that, taking into account that *F. armeniacum* is commonly associated with natural grasses in Argentina, the threat to animal health posed by this fungus should not be underestimated. Further studies should strive to completely characterise exposures due to contamination by this *Fusarium* species. Also, the simultaneous production of a broad range of type A trichothecenes, BEA, AUF and ZEA, often at high levels, assessed for most of *F. armeniacum* isolates under study is very important since the co-occurrence of different toxic metabolites could imply additive and/or synergistic effects on target organisms.

In conclusion, new data on the toxigenic profile of an important number of *F. armeniacum* isolates have been obtained, confirming that this fungus could be a potent producer not only of T-2 and HT-2, but also of other trichothecenes A of concern, such as NEO, DAS, MAS, and BEA which is considered an emerging *Fusarium* mycotoxin. Furthermore, the ability of the fungus to produce ZEA and AUF has been shown for the first time.

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