Aflatoxin production in three selected samples of triticale, wheat and rye grown in Argentina

Luis G Bilotti,† Virginia E Fernández Pinto and Graciela Vaamonde*
Laboratorio de Microbiología de Alimentos, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 3° Piso, 1428 Buenos Aires, Argentina

Abstract: Little is known about mycotoxin contamination of triticale, a hybrid resulting from crossing wheat and rye. The purpose of the present work was to evaluate triticale as a substrate for aflatoxin accumulation in comparison with its parents. Aflatoxin (B_1, B_2, G_1 and G_2) accumulation curves were obtained for the three substrates inoculated with *Aspergillus parasiticus* NRRL 2999 and incubated at 25°C and water activity 0.925 for 10 weeks. Wheat and triticale were poor substrates for aflatoxin production. Rye was more prone than the other substrates to fast colonisation by *A parasiticus* and accumulated larger aflatoxin quantities over the whole incubation period. The maximum aflatoxin concentration in rye (11840 $\mu g kg^{-1}$) was significantly larger ($p < 0.05$) than those obtained in wheat (2150 $\mu g kg^{-1}$) and triticale (2850 $\mu g kg^{-1}$).

Keywords: aflatoxins; wheat; rye; triticale

INTRODUCTION

Triticale is a hybrid resulting from crossing wheat and rye that combines many of the best characteristics of both prodigious parents. It has most of wheat's qualities for making various types of noodles, pastries and some breads and also has most of rye's disease resistance, drought tolerance and adaptability to harsh environments.[1] At the present time this cereal is considered a crop with promising perspectives in relation to the requirements of more and better foods.[2]

Little is known about mycotoxin contamination in this promising crop. The aim of this investigation was to evaluate triticale and its parents as substrates for aflatoxin production. Aflatoxins, potent hepatocancerigens produced by *Aspergillus flavus* Link, *A parasiticus* Speare and *A nomius* Kurtzman *et al*, were selected for the present study because of their importance as natural contaminants of foods and feeds, mainly cereals and oilseeds. Data on the natural incidence of aflatoxin contamination in wheat in several parts of the world indicate that it is not a very susceptible substrate, since aflatoxins levels detected in this cereal are generally low.[3–5] There are not many references to rye susceptibility, although it has been included among the small grains, as well as barley, oats, millet, sorghum and rice, which are less prone to contamination with significant levels of aflatoxins unless subjected to poor post-harvest handling.[4,6,7]

In the present work, aflatoxin accumulation curves were obtained by analysing aflatoxins B_1, B_2, G_1 and G_2 produced at selected times in cereals inoculated with *A parasiticus* NRRL 2999 and incubated under controlled environmental conditions for a 10 week period. Data from the accumulation curves allowed us to compare the behaviour of triticale, wheat and rye as substrates for aflatoxin production.

MATERIALS AND METHODS

Fungi

A parasiticus NRRL 2999 was used. The strain is a strong producer of aflatoxins B_1, B_2, G_1 and G_2.

Substrates

Samples (2 kg) of wheat (cultivar Bonaerense Valverde), rye (cultivar Don Lisandro INTA) and triticale (hybrid Yagan INTA) came from the Estación Experimental Agropecuaria Bordenave of the Instituto Nacional de Tecnología Agropecuaria (INTA) located in the Province of Buenos Aires, Argentina. The materials were treated by gamma irradiation[8] to kill contaminant micro-organisms while maintaining, as closely as possible, the natural condition of the grains. Irradiation was carried out using a ^{60}Co source at a dose rate of 6 kGy.[9] Water activity (a_w) was adjusted to 0.925 by adding sterile water to the irradiated grains.

* Correspondence to: Graciela Vaamonde, Laboratorio de Microbiología de Alimentos, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 3° Piso, 1428 Buenos Aires, Argentina
† Current address: Departamento de Biología Vegetal, Facultad de Biología, Universidad de Murcia, Campus del Espinardo, E-30100 Espinardo, Murcia, Spain
Contract/grant sponsor: Universidad de Buenos Aires
Contract/grant sponsor: Consejo National de Investigaciones Científicas y Tecnológicas (CONICET)
Contract/grant sponsor: Agencia National de Promoción Cientifica y Tecnológica
(*Received 19 January 2000; accepted 7 June 2000*)

Measurements of a_w were performed with an electronic hygrometer Vaisala Humicap HMI 31 with a sorption sonde HMP 35 calibrated with saturated salt solutions at 25 °C.[10]

Preparation of spore inocula

A parasiticus NRRL 2999 was grown on potato dextrose agar (PDA) for 7 days at 30 °C and washed with 5 ml of sterile glycerol–water solution of a_w 0.925. The suspension was stirred with a mechanical shaker (Vortex) for 2 min. A concentration of 10^7 spores ml^{-1} was obtained by diluting the original suspension. The number of spores in the inoculating suspension was assessed by the use of a haemacytometer (Thoma chamber).

Inoculation and incubation

Grains equilibrated at a_w 0.925 were distributed in sterile Petri dishes in portions of 25 g, and 1 ml of the spore suspension was sowed in each plate. The grains were thoroughly mixed to obtain a homogeneous distribution of the inoculum and incubated at 25 °C ± 0.5 °C. To avoid changes in a_w, the plates were placed above a saturated solution of KNO_3 (a_w 0.925) enclosed in polyethylene bags (gauge 0.04 mm). Three dishes of each substrate were taken every 7 days during 10 weeks for aflatoxin analysis.

Aflatoxin analysis

Aflatoxins were quantitatively determined using a technique based on the BF method (AOAC method 49.2.09),[11] with some modifications. Seeds (30 g) were extracted with 150 ml of methanol–4% KCl(aq) (90:10 v/v) in a high-speed blender (2 min). The extract was filtered through Whatman No 4 filter paper, and 50 ml of the filtrate was extracted with 25 ml of hexane in a separatory funnel. The lower aqueous methanol phase was transfered to a second separatory funnel, and 20 ml of 4% KCl(aq) was added. Aqueous methanol was extracted with 50 ml of $CHCl_3$ and collected through filter paper with Na_2SO_4(anh). The extraction was repeated with 30 ml of $CHCl_3$. Combined chloroformic extracts were evaporated to dryness using a rotary evaporator. The dry extract was dissolved in benzene–acetonitrile (98:2 v/v) for thin layer chromatography (TLC). Silica gel 60 plates (Merck Catalogue No 5725, 0.25 mm) were used in a non-saturated chamber at room temperature. The developing solvent was chloroform–acetone (90:10 v/v). Aflatoxin (AFB_1, AFB_2, AFG_1 and AFG_2) concentrations were determined by visual comparison of fluorescence under UV light (366 nm) with a standard solution (Sigma Chemical Co, St Louis, MO, USA) in benzene–acetonitrile (98:2 v/v). Total aflatoxin concentration was the sum of the four individual toxins at each point of time.

The method was assayed on samples of the three substrates spiked with aflatoxin B_1 at levels of 50, 100 and 200 $\mu g\,kg^{-1}$. Recovery at each level was expressed as a percentage. The relative standard deviation (RSDr) was calculated for each spiking level to give a measure of the precision of the method.

Statistical analysis

The limit of detection of the analytical method was calculated by linear regression, plotting aflatoxin spiked versus aflatoxin detected.

Analysis of variance (ANOVA) was performed to detect differences in maximum accumulation of each toxin obtained for the three substrates. The least significant difference (LSD) test was carried out for pairwise comparisons.

Statistix^R version 4.1 software (Analytical Software, USA) was used to analyse the experimental results.

RESULTS AND DISCUSSION

The proposed method for aflatoxin analysis in wheat, rye and triticale showed good performance for the three substrates (Table 1). Recoveries in excess of 90% for wheat at the three levels and higher than 86% at all levels in the case of rye and triticale were achieved. RSDr was lower than 20% in all cases, demonstrating good precision. The statistical limit of detection was 24 $\mu g\,kg^{-1}$ for wheat and 35 $\mu g\,kg^{-1}$ for rye and triticale. The limit of detection determined experimentally was 20 $\mu g\,kg^{-1}$ for the three substrates. Lower quantities could be detected but not quantified because of matrix interferences. Taking into account these analytical attributes, the method was considered appropriate for quantitative detection of aflatoxins in the three substrates.

Table 2 shows data of aflatoxin accumulation in each substrate after different incubation times at 25 °C and a_w 0.925. The largest quantities of aflatoxins B_1, B_2, G_1 and G_2 were accumulated in rye over the whole

Table 1. Recovery and precision results for aflatoxin B_1 (AFB_1) in wheat, rye and trititicale

	Wheat[a]			*Rye*[b]			*Triticale*[b]		
AFB_1 spiked ($\mu g\,kg^{-1}$)	*AFB_1 found ($\mu g\,kg^{-1}$)*	*Recovery (%)*	*RSDr*	*AFB_1 found ($\mu g\,kg^{-1}$)*	*Recovery (%)*	*RSDr*	*AFB_1 found ($\mu g\,kg^{-1}$)*	*Recovery (%)*	*RSDr*
50	46	92	19.44	46.66	93.3	12.37	46.66	93.3	12.37
100	92	92	9.09	86.66	86.6	13.23	86.66	86.6	13.23
200	186	93	11.78	183.33	91.6	15.74	91.6	91.6	15.74

[a] Five replicates.
[b] Three replicates.

	Rye				Wheat				Triticale			
Days	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4	59	ND	55	ND	Tr	ND	Tr	ND	Tr	ND	Tr	ND
7	604	Tr	1860	52	83	Tr	248	Tr	479	Tr	1395	94
14	1875	209	6510	291	333	Tr	852	33	833	65	1744	215
21	2083	109	3100	192	514	29	1550	58	514	61	970	62
28	2583	207	7052	353	450	25	747	47	833	143	1550	215
35	3250	259	7982	353	362	44	800	45	556	71	1163	153
42	2767	463	405	372	200	29	620	Tr	517	Tr	682	92
49	2500	238	4107	250	159	Tr	336	Tr	200	229	568	67
56	1222	136	703	Tr	150	ND	140	ND	230	Tr	504	46
63	562	Tr	120	Tr	122	ND	103	ND	228	Tr	480	45
70	231	ND	64	ND	117	ND	103	ND	222	Tr	430	44

Table 2. Aflatoxin production ($\mu g\,kg^{-1}$) in rye, wheat and triticale infected with *Aspergillus parasiticus* NRRL 2999 (a_w=0.925; T=25°C; inoculum, 10^5 spores$\,g^{-1}$)

All results are mean values of three replicates
ND, not detected; Tr, trace.

period analysed. Maximum accumulations for AFB_1 ($3250\,\mu g\,kg^{-1}$) and AFG_1 ($7982\,\mu g\,kg^{-1}$) were reached in this substrate after 35 days, while the maxima for AFB_2 ($463\,\mu g\,kg^{-1}$) and AFG_2 ($372\,\mu g\,kg^{-1}$) were reached after 42 days. Production of AFB_2 and AFG_2 was delayed, since these toxins only began to be detected after 7 days, while AFB_1 and AFG_1 were detected in 4 days. This effect was also observed in the other two substrates (Table 2) as well as in corn[12] and amaranth grain,[13] being explained on the basis of metabolic conversion of AFB_1 and AFG_1 to AFB_2 and AFG_2 respectively through the biosynthetic pathway. AFB_2 and AFG_2 were not detected in rye at the end of the period (70 days). Accumulation of the four aflatoxins in wheat was lower than in rye and similar to that observed in triticale. AFB_2 and AFG_2 were produced in wheat in very low quantities and they again appeared delayed. These toxins were not detected in wheat after 56 days of incubation, showing a higher rate of degradation after reaching the maximum accumulation.

The substrates can be compared taking into account the maximum quantities of aflatoxins accumulated in each of them. Analysis of variance demonstrated that maximum accumulations of AFB_1, AFB_2 and AFG_1 in rye were significatively higher ($p < 0.05$) than those observed in the other substrates (Table 3). There were no significant differences ($p < 0.05$) between maximum accumulations detected in wheat and triticale, except for AFG_2 which is produced in very low quantities in wheat. Table 2 shows that, in general, the maximum accumulation was detected earlier in triticale (after 14 days for AFB_1, AFG_1 and AFG_2) and wheat (21 days for AFB_1, AFG_1 and AFG_2) than in rye (35 days for AFB_1 and AFG_1 and 42 days for AFB_2 and AFG_2).

The marked difference in the behaviour of the two ancestors can be observed in Fig 1, which shows the total aflatoxin accumulation curves for each substrate

	Aflatoxin ($\mu g\,kg^{-1}$)			
Substrate	B_1	B_2	G_1	G_2
Wheat	514a	44a	1550a	58b
Triticale	833a	143a	1744a	215a
Rye	3250b	463b	7982b	372a

Table 3. Maximum accumulation of aflatoxins ($\mu g\,kg^{-1}$) in wheat, rye and triticale infected with *Aspergillus parasiticus* NRRL 2999 (a_w=0.925; T=25°C; inoculum, 10^5 spores$\,g^{-1}$)

Values are means of three replicates
Values followed by the same letter within the same column are not significantly different ($p < 0.05$) by the LSD test.

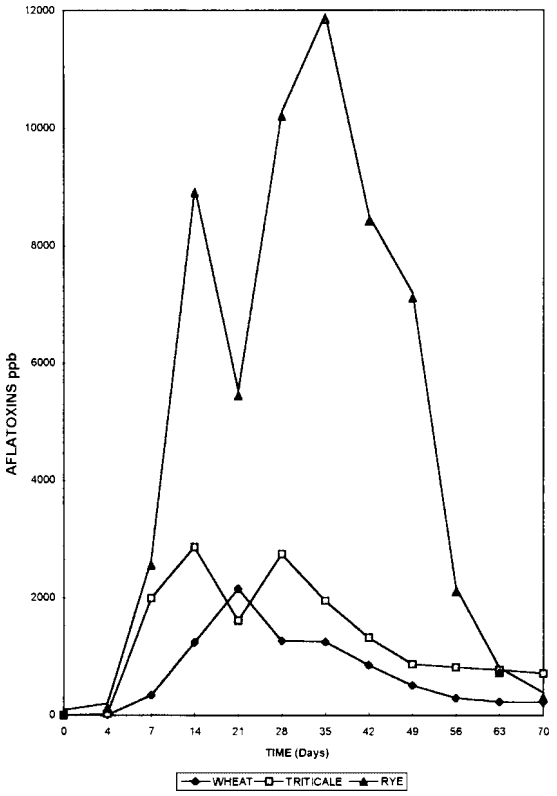

Figure 1. Total aflatoxin accumulation in wheat, rye and triticale infected with *Aspergillus parasiticus* NRRL 2999 (a_w=0.925; T=25°C; inoculum, 10^5 spores$\,g^{-1}$).

at 25 °C and a_w 0.925. Although rye is considered a rustic crop that resists diseases that affect wheat, in these experiments it proved to be more prone to fast colonisation by *A parasiticus* and accumulated larger quantities of aflatoxins under favourable environmental conditions. The accumulation curve for triticale showed a similar shape to that for rye, but aflatoxin accumulated quantities were lower and very similar to that observed in wheat.

Wheat and triticale showed a lower tendency to aflatoxin accumulation in comparison not only with rye but also with other cereals and oilseeds. Montani *et al*[14] carried out similar experiments with the same fungal strain inoculated in corn, sunflower and sorghum. Maximum total aflatoxin concentrations found in the present work in wheat (2150 $\mu g\,kg^{-1}$) and triticale (2850 $\mu g\,kg^{-1}$) are similar to that observed by Montani *et al*[14] in sorghum (2260 $\mu g\,kg^{-1}$), considerably lower than that in sunflower (15 720 $\mu g\,kg^{-1}$) and very low in comparison with corn (30 760 $\mu g\,kg^{-1}$) at the same a_w. These results seem to be in agreement with the observation that small grains, in general, are substrates less susceptible to aflatoxin contamination. Amaranth seeds, which are very small, are also a poor substrate for aflatoxin production.[13,15] According to our results, the behaviour of rye appears to be exceptional, because, in spite of having grains slightly smaller than those of triticale, the maximum total aflatoxin accumulation detected (11 840 $\mu g\,kg^{-1}$) would indicate a higher propensity for aflatoxin contamination. It is evident that the substrate factors involved in the contamination process are not much related to the size of the grains. It can be hypothesised that small grains could be dried faster than large ones because of their larger specific surface area, and, as a consequence, they would have the advantage of easier post-harvest management and a lower incidence of contamination in the food production chain. When temperature and a_w are optimal for fungal growth and aflatoxin biosynthesis, differences between substrates could be determined by factors other than the size of the grains. In this respect, the influence of some components such as sugars, certain metals, phenolic compounds and phytoalexins on aflatoxin biosynthesis has been pointed out.[5,16–18] Further studies on the differences in chemical composition of these cultivars of triticale, wheat and rye would be needed in order to support this view.

Results of the present work indicates that triticale, besides the good qualities mentioned above, could have the advantage of being relatively resistant to aflatoxin contamination like one of its ancestors (wheat), even taking into consideration that the results are only applicable to the cultivars investigated.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support of Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET) and Agencia Nacional de Promoción Científica y Tecnológica. GV is a member of CONICET.

REFERENCES

1 ACTI (Advisory Committee on Technology Innovation), *Triticale, a Promising Addition to the World's Cereal Grains. Report of an Ad-hoc Panel of the ACTI Board on Science and Technology for International Development, National Research Council*. National Academic Press, Washington, DC (1989).
2 Varughese G, Pfeiffer WH and Peña RJ, Triticale: a successful alternative crop. *Cereal Foods World*. **41**:635–645 (1996).
3 FAO, Perspective on mycotoxins, *Food and Nutrition Paper 13*, FAO and UNEP, Rome (1979).
4 Smith JE and Moss M, *Mycotoxins. Formation, Analysis and Significance*. Wiley, Chichester, pp 83–87 (1985).
5 Pittet A, Natural occurrence of mycotoxins in foods and feeds—an updated review. *Rev Med Vet* **149**:479–492 (1998).
6 CAST (Council for Agriculture Science and Technology), Mycotoxins. Economic and health risks. *Task Force Report 116*, pp 37–42 (1989).
7 FAO, Mycotoxin surveillance, *Food and Nutrition Paper 21*, FAO and UNEP, Rome (1982).
8 Cuero RG, Smith JE and Lacey J, The influence of gamma irradiation and sodium hypochloride sterilization on maize seed microflora and germination. *Food Microbiol* **3**:107–113 (1986).
9 Comerio R, Fernández Pinto VE and Vaamonde G, Influence of water activity on *Penicillium citrinum* growth and kinetics of citrinin accumulation in wheat. *Int J Food Microbiol* **42**:219–223 (1998).
10 Chirife J, Favetto G, Ferro Fontán C and Resnik SL, The water activity of standard saturated salt solutions in the range of intermediate moisture foods. *Lebensm Wiss Technol* **16**:36–38 (1983).
11 AOAC (Association of Official Analytical Chemists), *Official Methods of Analysis*, 16th ed., AOAC, Washington, DC, Chap 49, p 10 (1995).
12 Montani ML, Vaamonde G, Resnik SL and Buera P, Water activity influence on aflatoxin accumulation in corn. *Int J Food Microbiol* **6**:349–353 (1988).
13 Bresler G, Vaamonde G, Degrossi C and Fernández Pinto VE, Amaranth grain as substrate for aflatoxin and zearalenone production at different water activity levels. *Int J Food Microbiol* **42**:57–61 (1998).
14 Montani ML, Vaamonde G, Resnik SL and Martínez E, Comparison of capability of sorghum, sunflower and corn to support aflatoxin production at different levels of water activity. *VIII Int IUPAC Symp on Mycotoxins and Phycotoxins*, Mexico, p 71 (1992).
15 Fernando T and Bean G, Production of aflatoxin on amaranth seeds by *Aspergillus* species. *Food Chem* **17**:33–39 (1985).
16 Abdollahi A and Buchanan RL, Regulation of aflatoxin biosynthesis: induction of aflatoxin production by various carbohydrates. *J Food Sci* **43**:633–635 (1981).
17 Diener UL, Asquith RL and Dickens JW, Aflatoxins and *Aspergillus flavus* in corn. *Bulletin 279*, Department of Research Information, Alabama Agricultural Experimental Station, Auburn University (1983).
18 Failla LJ, Lynn D and Niehaus Jr WG, Correlation of Zn content with aflatoxin content of corn. *Appl Environ Microbiol* **52**:73–74 (1986).