Occurrence of *Aspergillus flavus* strains and aflatoxins in corn from Santa Fe, Argentina

Marcelo C. Nepote [1], Eduardo Piontelli L.[2] y Adriana Saubois [1]

SUMMARY. It has been demonstrated in several agricultural regions all around the world that *Aspergillus flavus* can infect corn grains and produce aflatoxins even before the harvest. It is also known that the incidence and levels of contamination of cereals factors. In the present work, the incidence of aflatoxins in corn grain from the central and northern areas of Santa Fe province in Argentina was studied. The relationship between the extent of kernel infection by the fungus and the presence of aflatoxins in the samples was examinated. The isolation and identification of *A.flavus* were carried out by plating dilutions of the ground kernels on dichloran-rose bengal-chloramphenicol agar (DRBC). Simultaneously, kernels were superficially sterilized with 10% commercial ClONa and plated on potato-dextrose-chloramphenicol agar (PDA+C). The analysis of aflatoxins B1, B2, G1 and G2 was performed by thin layer chromatography (TLC) according with Norma IRAM 14803 (Argentina). *A.flavus* Link:Fr. was identified in 63.3% of the corn samples. Colonized kernels ranged from 2.5 to 25% and counts on DRBC were in the order of 10^3 CFU/g. Two samples colonized by *A.flavus* contained aflatoxins B1 and B2 (50 µg/kg of aflatoxin B1 and 30 µg/kg of aflatoxin B2, and 30 µg/kg of aflatoxin B1, and traces of aflatoxin B2, respectively). One sample contained only aflatoxin B1 (22 µ/kg). According to these results, it may be concluded that the incidence of *A.flavus* observed constitutes a call in attention with respect to the conditions required for storage and transportation of the grains, to minimize the proliferation of the fungus and the production of aflatoxins in these stages. Although the incidence of aflatoxins in the samples of grains was rather low, the levels of aflatoxin B1 recorded in the positive samples were higher than those recommended -or given as adivsory levels for human foods, by most countries in the world.

RESUMEN. Incidencia de cepas de *Aspergillus flavus* y aflatoxinas en maíz de Santa Fe, Argentina. *Aspergillus flavus* puede infectar los granos de maíz y producir aflatoxinas aún antes de la cosecha en determinadas áreas agrícolas. La incidencia y niveles de contaminación de las mismas varían año a año. Se estudió la incidencia de aflatoxinas de muestras de granos de maíz suministradas por productores de la zona centro-norte de la provincia de Santa Fe, Argentina. Se examinó la relación existente entre la infección de los granos por *A.flavus* y la presencia de aflatoxinas en las muestras. El aislamiento e identificación de *A.flavus* se realizó a partir de las diluciones de grano molido, sembrado en agar diclorán-rosa de Bengala-cloranfenicol (DRBC). Simultáneamente, los granos enteros fueron desinfectados superficialmente con ClONa comercial 10% y sembrados sobre agar papa-dextrosa-cloranfenicol (PDA+C). El análisis de aflatoxinas B1, B2, G1 y G2 se llevó a cabo por cromatografía en capa fina (TLC) según Norma IRAM 14803, Argentina. La confirmación de las mismas se realizó mediante TLC bidimensional. Se identificó *A.flavus* Link:Fr en el 63,3% de las muestras, con un porcentaje de granos contaminados entre el 2,5 y 25%, y un recuento en DRBC del orden de 10^3 UFC/g. De las muestras contaminadas con *A.flavus*, 2 contenían aflatoxinas B1 y B2 (50 µg/kg y 30 µ/kg y 30 µg/kg y trazas, respectivamente), y una muestra contenía sólo aflatoxina B1 (22 µ/kg). Se concluye que la incidencia de *A.flavus* constituye una alarma en cuanto a las condiciones requeridas para el almacenamiento y transporte de estos granos, como causa posible de la proliferación de la especie y la producción de aflatoxinas. Aunque la incidencia de aflatoxinas en las muestras resultó baja, los niveles de aflotoxina B1 determinados estuvieron por encima de las tolerancias para alimentos en la mayoría de los países.

INTRODUCTION

Corn grain is widely consumed in Argentina as grinding by-products which are also employed to manufacture several staple foods including snacks, popcorn, pies and other regional foods. It is one of the agricultural products of major interest as regards to its susceptibility to become contaminated with aflatoxins (1,2). *Aspergillus flavus* can infect corn ears before and after harvesting has taking place, thus affecting nutritional and technological properties of the grains due to the fungal colonization (3). Consequently, aflatoxins may be produced by the fungus in all the stages from the field to the storage environment. Agronomical practices and insects as well as storage conditions (relative humidity, temperature, etc) play important roles in the formation of aflatoxins. Depending on the ability of the fungus to produce aflatoxins, biological and environmental conditions can favour increasing of aflatoxins concentration after harvest (3,4,5,6).

Aflatoxins may induce adverse economical effects such as decreasing of agricultural, cattle and poultry yields. These losses may be due not only to an increase in death rate, but also to subacute toxicity effects (immunosupression, lower growing, rate, etc) (3,4,5,6).

Usually, a direct relationship can be established between aflatoxins incidence and *A.flavus* occurrence in raw materials, being highest contamination recorded in tropical and subtropical geographical areas. The climatic phenomena known as drought/temperature stress as weel as nitrogen deficiency in agricultural soils can enhance the problem and, if situacion gets to an extreme condition, most part of the crop can become contamined with aflatoxins (3,4).

Consequently, the present research was carried out on the natural occurrence of aflatoxins in whole corn grain samples from the province of Santa Fe, Argentina. Results were compared with those obtained from a previous survey in the same geographical area. In addition, the relationship between grain colonization by *A.flavus* and the incidence of aflatoxins in the samples was examined. In order to obtain a very fair representation of the contamination with the species, a combination of dilution plate counts and direct plating after

1. Investigador, Codirector de Proyecto de la Universidad Nacional del Litoral. Cátedra de Microbiología, Dpto.de Biotecnología, Facultad de Ingeniería Química, Santa Fe, Argentina. Investigadora, Directora d Proyecto de la Universidad Nacional del Litoral, Santa Fe, Argentina.
2. Profesor Investigador de la Universidad de Valparaíso, Chile. Cátedra de Micología, Escuela de Medicina, Universidad de Valparaíso. Chile.

surface desinfection was carried out, representing surface and internal contamination respectively.

MATERIAL AND METHODS

Samples: Thirty samples of whole corn grain from the central and northern regions of Santa Fe province in Argentina, corresponding to 1993 crop, were analyzed. Half of the total came directly from the field; the rest of the samples underwent post-harvesting dryingprocess. Sample weights were among 1.2 and 1.5 kg. Each sample was divided in 2 portions: 1) 200 g of whole grain were aseptically separated and stored at 0 °C in order to be evaluated for their contamination with *A.flavus*; 2)the remaining fraction was milled and quartered, preserving a portion of 200 g for aflatoxin analysis.

Enumeration and identification of *Aspergillus flavus:* Enumeration was carried out both by dilution plating and direct plating. Dilution plating method was performed on dichloran-rose Bengal-chloramphenicol agar (DRBC). Plates were incubated at 25 °C for 5 days. The results were expressed as colony-forming units per gram of sample (CFU/g) (7,8). Total fungal counts as well as *A.flavus* counts were recorded. The later were deduced from the total by recognition of *A. flavus* characteristic colonies with a stereomicroscope (8,9).

Direct plating was performed on pap-dextrose-chloramphenicol agar (PDA+C). Approximately 100 kernels/sample were previously surface desinfected with commercial sodium hypochlorite (final concentration 0.4%) and rinsed with sterile water, using about the same volume as the chlorine. After drying kernels with sterilized absorbent paper, 6 to 8 pieces were placed onto each of 10 solidified PDA+C containing plates. They were incubated at room temperature (22 to 25 °C) for 5 days. The results were expressed as percentage (%) of contaminated particles (kernels). Direct plating provides an estimate of the extent of infection of grains (7).

Fungal colonies were previously recognized according to their conspicuous macroscopic and microscopic features directly on enumeration plates. *A.flavus* presumptive colonies were isolated and identification was confirmed on malt extract agar (MEA) and Czapek-yeast extract agar (CYA) (10,11,12).

Aflatoxin analysis: It was carried out by thin-layer chromatography (TLC) according to Normas IRAM 14803, Argentina (13). Detection and quantification of aflatoxins B1, B2, G1 and G2 was performed by sprying with 30% $SO4H_2$ and by means of two-dimensional TLC (13,14). Standard of aflatoxins B1, B2, G1 and G2 were purchased from Sigma Chemical Co.

Moisture content: Moisture contens are given in a wet weight basis, calculated as (mass of water/total mass) x 100%. They were obtained by indirect method, in a laboratory oven at 100 °C, up to constant weight (approximately 24 h to 36 h).

RESULTS AND DISCUSSION

All the samples gave total fungal counts on DRBC in the order of 10^4 CFU/g ($1x10^4$ to $7.5x10^4$ CFU/g, mean $1.9x10^4$ CFU/g). According to APHA, total fungal counts ranging from 10^4 to 10^4/g are considered «normal» grains in commercial channels (15). No special standards are given for total fungal counts in cereals by different countries. Recommended levels are frequently dependent on the type of grain and the destination of the crop (human food or animal feed). As an example, some authors concluded that mould counts of feed should not exceed range of 10^4 counts/g (16).

Total fungal counts were one order higher than *A.flavus* count on DRBC in those samples where the species was detected. The results concerning enumeration and identification of *A.flavus* as well as the occurrence of aflatoxins and moisture contents of the samples are shown in Table 1. *A.flavus* Lin:Fr was identified in 63,3% of the samples. Counts on DRBC ranged from $1x10^3$ to $5x10^3$ CFU/g (mean $1.4x10^3$ CFU/g) and percentage of infected kernels varied from 2.5 to 25% (mean 14.7%). Only in 4 samples the species was detected by direct and dilution plating. In 3 of the samples the species was detected only by direct plating after surface desinfection. No relationship was observed between *A.flavus* counts on DRBC and percentage of infected kernels with the species. This was coincident with observations from previous collaborative studies carried out on corn and other cereal grains (8). Nevertheless, they are also coincident with the fact that determination of percentage of infection of sample with a particular fungus, together with dilution plate counts, gives a more detailed description on the distribution of the species. This has a special sense when a toxigenic fungal species is under consideration.

TABLE 1
Incidence of *A.flavus* and aflatoxins in corn grain

Sample N°	% Kernels infected with *A.flavus*(mean)	*A.flavus* counts $x10^3$ (CFU/g)(mean)	Aflatoxin (µg/kg) B1	B2	Moisture content (%w.b) (mean)
1	25	2	50	3	10.0
1	8	1	22	nd	11.5
1	7.5	1	30	tr	14.6
3	2.5-15.6 (7.7)	nd	nd	nd	13.0-15.0(13.7)
1	nd	1	nd	nd	12.0
13	2.5-22.9 (6.7)	1-5 (1.5)	nd	nd	11.0-14.1 (13.0)
10	nd	nd	nd	nd	11.2-15.0 (13.2)

% w.b.: moisture content expressed as percentage in wet basis
CFU/g: colony-forming units per gram of sample
n.d: not detected
B1: aflatoxin B1 B2: aflatoxin B2
tr: traces

Alfatoxins were detected in only 3 samples of the total (10 %), one sample containing only aflatoxin B1. Aflatoxin G1 and G2 were not detected. Even though aflatoxin incidence was low, the aflatoxin B1 levels were above the tolerance limit for foodstuffs adopted by most countries (5-20 µg/kg) (17,18,19). Present results are comparable with those obtained from a previous study on the incidence of aflatoxins in corn grains of the same geographical region (20). On that occasion, aflatoxins had been detected in 22 % from a total of 100 samples.

Because of low incidence of aflatoxins in the samples, no clear relationship could be established between the toxin level and either the percentage of infected kernels or *A.flavus* counts in the samples. However, samples with a relatively high percentage of contaminated grains (12.5 to 22.9%) did not contain aflatoxins in detectable levels. This might reflect a similar situation to that observed in certain areas of the world where a high incidence of *A.flavus* is observed every year but of which aflatoxigenic potential is relatively or definitely low. As an illustration of this, in drought years aflatoxin in corn can be an important problem in the southeastern United States, but is seldom a problem in Minnesota (4,21). It becomes apparent that rather special conditions are required for appreciable amounts of aflatoxins to be produced.

Moisture content of the substrate and relative humidity are critical factors to aflatoxin production in cereal grains. Though both parameters should be considered together in evaluating resistance to aflatoxin contamination, only moisture content was determined in these samples due to the fact that it constitutes one fo the main parameters for commercialization of cereal grains in the region. This values relates to a relative humidity at a given temperature, which characterize the atmosphere in equilibrium with the grain (22). The moisture contents obtained for present samples ranged from 11.0 to 15.0% (mean 12.6%). According to the literature, these samples could be considered secure only at a relative humidity below 82% from the point of view of aflatoxin production in corn (3,21). But levels near 15.0% of moisture content may not be adequate for long-term safe storage, since only small changes in aw may be necessary to allow large increases in fungal activity (1). On the other hand, the relatively high incidence of *A.flavus* in the samples, even after grain drying, contitute a warning sign to be considered in the subsequent storage, processing and transport stages.

CONCLUSIONS

- Since 63.3% of the samples gave positive results for *A.flavus* incidence by means of direct plating after surface desinfection, this methodology is considered preferable in recovering the species from corn grains.
- Corn grain is a special source of contamination with *A.flavus* in the region. Despite the relatively high incidence of the species, aflatoxin occurrence in the samples was low in terms of frequence and levels of contamination. This was observed in two studies made in the same sampling area corresponding to different years of harvest. Nevertheless, repetitive sampling should be carried out every year for aflatoxin analysis since unusual drought-stress and hot wether conditions may enhance -or even induce the ability of *A.flavus* to produce toxins in corn (21).
- More research is nedeed to understand the physiology, metabolism and nutritional requirements of aflatoxigenic strains of *A.flavus* from the region.
- Due to the above conclusions, central and northern regions of Santa Fe province in Argentina should not yet be considered of low risk as regards economical or public health aspects of aflatoxin contamination.

ACKNOWLEDGMENTS

This work was supported by grants from the Programme «Curso de Acción para la Investigación y el Desarrollo» -Secretaría de Ciencia y Técnica, and the Fundación Facultad de Ingeniería Química, both from the Universidad Nacional del Litorial, Argentina. Authors thank the staff of the Cátedra de Inglés Técnico, Facultad de Bioquímica y Ciencias Biológicas of the Universidad Nacional del Litoral for their cooperation in translating the present article into its English version.

REFERENCES

1. Lacey J., Ramakrishna N., Hamer A., Magan N. & Marfleet I.C. Grain Fungi. In: Handbook of Applied Mycology. Foods and Feeds, Vol. 3 D.K. Arora, Mukerji K.G., Marth E.H. (Eds). Marcel Dekker, Inc New York. p.121-177. 1991.
2. Lacey J. Pre and post-harvest ecology of fungi causing spoilage of foods and other stored products. Journal of Applied Bacteriology Symposium Supplement. 11S-25S. 1989.
3. Gourama H., Bullerman L.B. *Aspergillus flavus* and *Aspergillus parasiticus* Aflatoxigenic fungi of concern in foods and feeds: A review. Journal of Food Protection 58(12):1395-1404, 1995.
4. Moss M.O. Mycotoxins of *Aspergillus* and other filamentous fungi. Journal of Applied Bacteriology Symposium Supplement 69S-81S. 1989.
5. Brown R.L., Cotty P.J., Clevelan T.E., Winstrom N.W. Living maize embryo influences accumulation of aflatoxin in maize kernels. Journal of Food Protection 56(11):967-971. 1993.
6. Council for Agricultural Science and Tecnology. Mycotoxins. Economic and Health Risks. Task Force Report No. 116 (USA), Nov 1989.
7. Pitt J.L. & Hocking A.D. Fungi and food spoilage. Academic Press, Australia, 1985.
8. King A.D., Jr., Pitt J.L., Beuchat L.R. & Corry JEL. Methods for de Mycological Examination of Foods. Plenum Press, New York, 1986.
9. Williams A.P. Methodological development in food mycology. Journal of Applied Bacteriology Symposium Supplement. 61S-67S. 1989.
10. Raper K.B. & Fennel D.L. The Genus *Aspergillus*. The Williams & Wilkins Co. Baltimore., USA. 1965.
11. Klich M.A., Pitt J.I. Distinguishing species of the *Aspergillus flavus* Group. In: Advances in *Penicillium* and *Aspergillus* Systematics. R.A. Samson and J.I. Pitt (Eds.). Plenum Press, New York, p.211-220. 1985.
12. Klich M.A., Pitt. J.I. Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. Trans. Br Mycol. Soc. 91:99, 108, 1988.
13. Instituto Argentino de Racionalización de Materiales. Método de determinación de aflatoxinas y zearalenona en maíz y maní. Norma IRAM 14803, Argentina, 1990.
14. Association of Official Analytical Chemists. Natural Poisons. In: Official Methods of Analysis, 15th Ed. AOAC International, USA. p.1184-1213. 1990.
15. Mayou J. & Moberg L. Cereal and cereal products. In: Compendium of Methods for the Microbiological Examination of Foods. Third Ed. C. Vanderzant & D.F. Splittstoesser (Eds) of the American Public Health Association. Copyright by the APHA, Washington, DC. p.995-1006. 1992.
16. Trojanowska K. Evaluation of cereal grain using mycological methods. In: Cereal Grain. Mycotoxins, Fungi and Quality in Drying and Storage. J. Chelkowski (Ed). Elsevier, Amsterdam. p.217-227. 1991.
17. Código Alimentario Argentino. Capítulo XIX. Harinas, concentrados, Aislados y Derivados Proteicos. Res. M.B.S. 126,29-01-80, Argentina 1980.
18. Van Egmond H.P. Current situation on regulation ofr mycotoxins. Overview of tolerance and status of standards methods of sampling and analysis. Food Additives and Contaminants, 6(2):139-188, 1989.
19. Mercosul. Regulamento Técnico sobre Límites Máximos de Aflatoxinas. MERCOSUL/GMC/RES N° 56/94.
20. Nepote M.C., Saubois A., Beccaria A., Basilico J.C. Grado de contaminación por aflatoxinas y zearalenona en maíz y subproductos procesados en un molino harinero de la ciudad de Santa Fe (Argentina). Revista Iberoamericana de Micología 11:37-39. 1994.
21. Christensen C.M. Fungi and Seed Quality. In: Handbook of Applied Mycology. Foods and Feeds Vol 3. D.K. Arora K.G. Mukerji E.H. Marth (Eds). Marcel Dekker, Inc. New York p.99-120. 1991.
22. Bruce Dd.M., Ryniecki A. Economic methods of cereal grain drying to prevent spoilage and loss of quality. In: Cereal Gran. Mycotoxins, Fungi and Quality in Drying and Storage. J. Chelkoski (Ed.). Elsevier, Amstedrdam p.477-527. 1991.

Recibido: 10-09-1996
Aceptado: 18-04-1997