Journal of Food Protection, Vol. 71, No. 6, 2008, Pages 1262–1265

Research Note

Alternaria Toxins in Wheat during the 2004 to 2005 Argentinean Harvest

M. P. AZCARATE,[1] A. PATRIARCA,[2]* L. TERMINIELLO,[3] AND V. FERNÁNDEZ PINTO[2]

[1]*Instituto Nacional de Tecnología Agropecuaria. Estación Experimental Agropecuaria Anguil "Ing. Agr. Guillermo Covas.," Ruta Nacional 5 Km 580, C.C: 11 (6326), Anguil, La Pampa, Argentina;* [2]*Universidad de Buenos Aires, Departamento de Química Orgánica. Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria, Pabellón II, 3º Piso, CP 1428, Buenos Aires, Argentina; and* [3]*Universidad Nacional de La Plata, Facultad de Ciencias Agrarias y Forestales, Calle 60 y 119, CP 1900, La Plata, Buenos Aires, Argentina*

MS 07-508: Received 23 September 2007/Accepted 27 January 2008

ABSTRACT

The natural occurrence of *Alternaria* mycotoxins in Argentinean wheat from the zone 5 South during the 2004 to 2005 harvest was investigated in 64 wheat samples. All samples were highly contaminated with a wide range of fungal species. *Alternaria* was found as the main component of the mycota, with an infection percentage of 100%. Three mycotoxins produced by species of *Alternaria* were determined in wheat: alternariol, alternariol monomethyl ether, and tenuazonic acid. Alternariol was detected in 4 (6%) of 64 samples, with a range of 645 to 1,388 μg/kg (mean of 1,054 μg/kg); alternariol monomethyl ether, with a range of 566 to 7,451 μg/kg (mean of 2,118 μg/kg) in 15 (23%) of 64 samples; and tenuazonic acid in 12 (19%) of 64 samples, with a range of 1,001 to 8,814 μg/kg (mean, 2,313 μg/kg). Alternariol monomethyl ether was the predominant toxin, but tenuazonic acid was detected in higher concentrations. Alternariol was present in fewer samples and in lower levels than were the other toxins. Tenuazonic acid and alternariol monomethyl ether occurred together in four samples, while tenuazonic acid and alternariol co-occurred in one sample. This the first report of the natural occurrence of *Alternaria* mycotoxins in Argentinean wheat. Toxin levels were high, probably due to the heavy infection with *Alternaria* species found in the samples.

Wheat is one of the most important cereal crops in Argentina, both for local consumption and as an export commodity, with yields over 16 million tons (14.5 million metric tons) *(3).* The cultivated area is distributed according to agrometeorological conditions into five zones (Fig. 1b), with a main production area on the Buenos Aires Province (zones II South, IV South, and V South), east of La Pampa Province (zone V South) and south of Santa Fe Province (zone II North). This extended area presents very different conditions of temperature and humidity.

Alternaria species produce several mycotoxins belonging to three different structural classes: (i) tenuazonic acid (TA), a tetramic acid derivative; (ii) alternariol (AOH), alternariol monomethyl ether (AME) and altenuene, which are dibenzopyrone derivatives; and (iii) altertoxins I, II, and III, which are perylene derivatives *(5).* These toxins showed cytotoxic activity to bacterial and mammalian cells, and fetotoxicity and teratogenicity to mice and hamsters *(25).* AME and AOH are not very acutely toxic, with a 50% lethal dose higher than 400 mg/kg for mice, but show synergistic effects *(7),* and they are mutagenic in microbial and mammalian cell systems. There is also some evidence of carcinogenic properties *(1, 13, 23).* TA is not mutagenic in bacterial systems. However, it is toxic to several animal species; in dogs, it caused hemorrhages in several organs, and subacute toxicity in chickens was observed *(22).* Precancerous changes were observed in esophageal mucosa of mice fed 25 mg/kg of body weight per day of TA for 10 months *(27).* Additional toxicological studies are clearly needed. Although *Alternaria* is one of the major fungal genera found on grain, the presence of *Alternaria* mycotoxins has been largely ignored in these products *(26).* There are no specific regulations for any of the *Alternaria* toxins in food.

Alternaria toxins have been found to be natural food contaminants in grains, sunflower seeds, and some visibly decayed fruits in many countries *(2, 20, 23, 24).* Their natural occurrence in wheat has been reported in different countries *(12, 15, 26).* Several studies have reported the relevance of this genus in Argentinean wheat. González et al. *(9)* found that *Alternaria* was the second genus predominant in this crop. In more recent studies *(4, 8), Alternaria* was found as the major component of the wheat mycota. However, there are no studies on the natural occurrence of *Alternaria* toxins in Argentinean wheat.

Since *Alternaria* is a frequent fungal genus invading wheat, and considering its toxigenic capacity, the presence of its mycotoxins should be evaluated in order to determine a potential risk to consumer health. The purpose of this study was to evaluate the natural occurrence of *Alternaria*

* Author for correspondence. Tel. and Fax: +54-11-45763346; E-mail: andreap@qo.fcen.uba.ar.

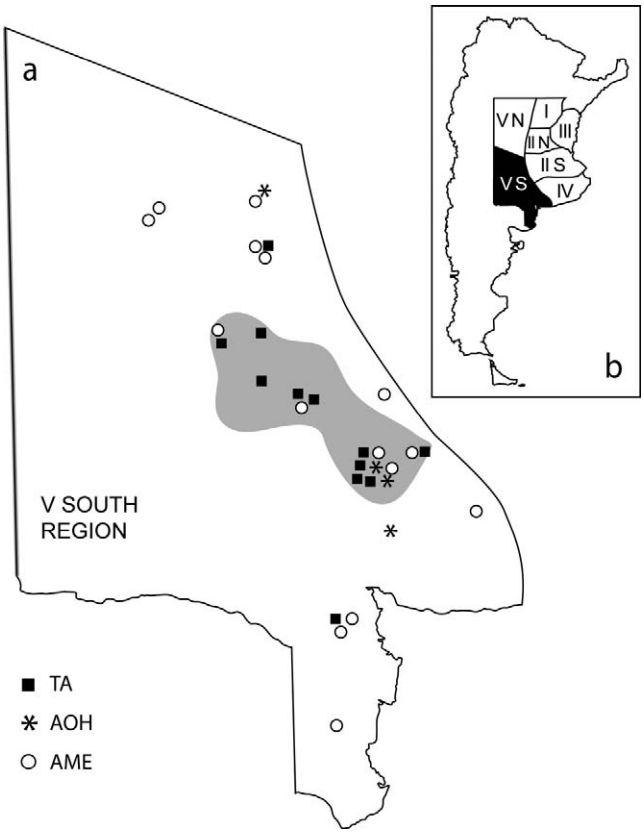

FIGURE 1. *(a) Map of the wheat production area VS, showing the location of positive samples for* Alternaria *mycotoxins during the 2004 to 2005 Argentinean harvest. The shaded area corresponds to the zone of maximum rainfall accumulated along the harvest. (b) Map of Argentina showing the five wheat-production regions and subregions (I, II, III, IV, and V). N, North; S, south.*

mycotoxins in wheat during the 2004 to 2005 Argentinean harvest.

MATERIALS AND METHODS

Wheat samples. Sixty-four samples of common wheat (*Triticum aestivum*) from the wheat production area known as V-South (VS) (east of La Pampa Province and southwest of Buenos Aires Province) were analyzed. The samples were collected from farms in 25 districts of the region during the 2004 to 2005 harvest (December 2004 through January 2005). Sampling was performed at ''Chacra Experimental Integrada Barrow'' and Estación Experimental Agropecuaria Bordenave (Instituto Nacional de Tecnología Agropecuaria), and the samples were collected soon after harvest. The kernels were harvested at maturity with 14% of moisture. Samples were at least 1 kg in size, not sorted by quality (all grades included), and were maintained at −30°C until analysis.

Fungal examination and identification. For the isolation of the internal mycoflora, subsamples of wheat kernels were superficially disinfected in a 10% aqueous solution of commercial sodium hypochlorite for 1 min, rinsed with sterile distilled water, submerged in a 70% ethyl alcohol solution and dried over a filter paper. A total of 100 kernels per subsample were placed, 10 per plate, on potato dextrose agar and incubated for 5 days at 25°C. The resulting fungal colonies were enumerated. Where several different fungi were isolated from a single kernel, all were recorded.

After subculture and purification from the primary isolation plates, identification was carried out using the methods and media of Pitt and Hocking *(17),* with reference to other mycological texts when necessary, especially Samson et al. *(18).*

Mycotoxin analysis. Each sample was ground in a laboratory mill and then a representative subsample was collected for analysis. The extraction method for *Alternaria* toxins in wheat was a modified procedure based on that described by Li and Yoshizawa *(12).* Ground wheat subsamples (15 g) were extracted with acetonitrile and 4% KCl (9:1, 75 ml) for 30 min, followed by the addition of 1 N HCl (15 ml). The mixture was filtered, and 45 ml of the filtrate (equal to 7.5 g of wheat sample) was initially clarified with 90 ml of 0.05 M lead acetate, and then filtered again. The filtrate was divided into two parts. The first part (75 ml) was extracted three times with 20 ml of chloroform. The organic phases were combined, evaporated to dryness, and dissolved in 4 ml of methanol for AOH and AME analysis by high-performance liquid chromatography (HPLC). The second part (36 ml, equal to 2 g of sample) was adjusted to pH 2 with 6 N HCl, filtered again, and extracted twice for TA with 25 ml of chloroform. TA was then partitioned into 15 ml of 5% sodium bicarbonate, acidified to pH 2.0 again, and extracted twice with chloroform (15 ml). The chloroform extracts were combined, washed with 13 ml of water, and evaporated to dryness. The residue was made up to 4 ml with methanol and analyzed for TA by HPLC.

The HPLC system consisted of a Shimadzu LC-CA liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Rheodyne sample valve fitted with a 20-μl loop and a Shimadzu SPD-M10Avp UV photodiode array detector. The analytical column was Jupiter, C18, 5 μm, 4.6 by 250 mm (Phenomenex, Torrance, Calif.). Standards of TA, AME, and AOH were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo.). The mobile phase was methanol and water (80:20) containing 300 mg/liter $ZnSO_4 \cdot H_2O$, for AOH and AME, and methanol and water (85:15) containing 300 mg/liter $ZnSO_4 \cdot H_2O$, for TA. A flow rate of 0.4 ml/min was used. The wavelength for recording chromatograms was 258 nm for AME and AOH and 280 nm for TA. A calibration curve was constructed for quantification purposes, using the toxin standards and correlating peak area versus concentration (levels of 100 to 1,000 μg/kg). Confirmation of all toxins was achieved by using a photodiode array detector. The spectra were acquired in the range of 200 to 400 nm on the apex and on the ascending or descending part of each peak, using a pilot signal at 258 nm for AOH and AME, and at 280 nm for TA. Reference spectra were acquired during the elution of associated standards and used for peak identification by comparison after spectra normalization.

The method detection limits were 80 μg/kg for TA and 50 μg/kg for AME and AOH. Mean recoveries of AOH and AME from triplicate spiked samples were 86 and 94%, with coefficients of variation of 4.76 and 6.13%, respectively, at a level of 500 μg/kg, and 91 and 97% at a level of 1,000 μg/kg, with coefficients of variation of 3.02 and 2.15, respectively. For TA, at a level of 500 μg/kg, the recovery was 70% with a coefficient of variation of 4.51%, and at a level of 1,000 μg/kg, the recovery was 78%, with a coefficient of variation of 1.96%.

RESULTS AND DISCUSSION

Fungal incidence in wheat samples. The distribution of genera in wheat from one of the principal wheat regions of Argentina in 2004 to 2005 is shown in Table 1. The level of fungal contamination of the wheat samples was very high, with an internal fungal infection between 80 and 100%. All samples presented a wide range of different fungal species, with *Alternaria* being the predominant genus,

TABLE 1. *Fungal incidence in wheat samples from the 2004 to 2005 Argentinean harvest*

Genus	No. of isolates[a]	% of isolates[b]	Contaminated samples (%)[c]
Alternaria	4,107	93	100
Epicoccum	155	3.5	80
Fusarium	54	1.2	46
Trichothecium	39	0.9	8
Chaetomium	16	0.4	22
Sordaria	16	0.4	14
Other genera	28	0.6	8

[a] Total number of isolates from 64 samples of wheat.
[b] (Number of isolates of each genus)/(total number of isolates) × 100.
[c] (Number of samples with occurrence of each genus)/(total number of samples) × 100.

with an infection percentage of 100%. *Epicoccum* and *Fusarium* spp. were also found as contaminants of a high number of samples, although in a lower proportion than *Alternaria* was. Other genera isolated with less frequency were *Chaetomium, Sordaria,* and *Trichothecium.*

In the present work, the genus *Alternaria* was found as the main component of the mycota in wheat from the VS region. These results are in agreement with those of González et al. *(8)* on Argentinean durum wheat. Studies in other countries have also reported a high incidence of this genus in wheat *(12, 26).*

Throughout the growing season, weather conditions such as temperature and humidity influence the distribution of the infecting fungal species and lead to geographical variation in the species distribution. Other important factors affecting fungal distribution are fungal interactions, including spatial competition between *Alternaria* and *Fusarium* species *(11). Alternaria* species isolated from grain have been shown to produce mainly TA, AOH, and AME *(6, 14, 19).* In a recent study, 123 *Alternaria* strains isolated from Argentinean wheat from the VS region were tested for mycotoxin production. All strains but one were able to synthesize at least one of these toxins, *A. tenuissima* and *A. alternata* being the most frequent species isolated *(16).*

Mycotoxin occurrence. The natural occurrence of *Alternaria* mycotoxins in Argentinean wheat from the VS region in the 2004 to 2005 harvest is shown in Table 2. The geographic distribution of positive samples is shown in Figure 1a.

AME was the predominant toxin, but TA was detected in higher concentration. AOH was present in fewer samples and in lower levels than were the other toxins. With regard to the co-occurrence of *Alternaria* toxins in wheat, TA and AME occurred together in four samples, while TA and AOH co-occurred in one sample, and no co-occurrence of AOH and AME was found.

Li and Yoshizawa *(12)* reported that TA was the major toxin in samples of weathered wheat from China (average of 2,419 μg/kg), and AME was found at higher levels than was AOH. The levels of TA agreed with those found in the present work, although AME was the major toxin present in Argentinean wheat, and AME and AOH were found in higher levels. AOH was present as a major toxin in Australian weathered wheat, ranging in concentration between 10 and 1,050 μg/kg; AME and TA were also detected at much lower levels *(26).* Only AOH (590 μg/kg) was found in wheat kernels from Poland, and the wheat was free from other *Alternaria* mycotoxins *(10).* AOH and AME were reported in German wheat (up to 200 and 12 μg/kg, respectively), and AOH, AME, altertoxin I, altenuene, and TA in Egyptian wheat (up to 2,300, 1,900, 1,700, 1,500, and 700 μg/kg, respectively) *(21).*

TABLE 2. *Occurrence of* Alternaria *mycotoxins in wheat from the 2004 to 2005 Argentinean harvest*

Mycotoxin[a]	No. of positive samples	% of positive samples[b]	Range (μg/kg)	Mean (μg/kg)
TA	12	19	1,001–8,814	2,313
AOH	4	6	645–1,388	1,054
AME	15	23	566–7,451	2,118

[a] TA, tenuazonic acid; AOH, alternariol; AME, alternariol monomethyl ether.
[b] A total of 64 samples were analyzed.

TA levels detected in Chinese and Argentinean wheat were much higher than those in other grains, such as sorghum and ragi reported previously *(2, 10, 26).* Natural occurrence of *Alternaria* mycotoxins in small grains such as sorghum, ragi, and sunflower seeds is well known, but there have been only limited reports in wheat *(25).* To our knowledge, this is the first report of the natural occurrence of these mycotoxins in Argentinean wheat. Although toxin levels are remarkably high, this could be due to the heavy infection with *Alternaria* species found in the samples.

So far, there have been very limited reports in the literature of the co-occurrence of these toxins in wheat, and they are without significant correlation in concentrations *(10, 26).* Li and Yoshizawa *(12)* detected the co-occurrence of TA and AME in 21 (95.9%) samples of weathered wheat from China, whereas AOH, AME, and TA occurred together in 20 samples (90.9%). The co-occurrence of *Alternaria* mycotoxins in Argentinean wheat was less frequent than in Chinese wheat, and no co-occurrence of the three toxins was detected. Differences in geographical and environmental conditions might be responsible for these differences in mycotoxin distribution and concentrations.

In wheat from the VS region in the 2004 to 2005 Argentinean harvest, the presence of the three mycotoxins was more significant in the southeast area and decreased toward the west and north, with an evident accumulation in the central zone of the region (Fig. 1a). The whole region had a wetter climate than usual during that period. At the beginning of the wheat-growing season, the precipitation was below the historic average in this region. Thus, the sowing of long cycle wheat began with scarce rainfall in May and June. In the months of August and December, the crop received heavy rainfall, especially in the area where *Alternaria* toxins were concentrated. The shaded area in Figure

1a represents the zone of maximum rainfall accumulated during those months *(3)*. It could be hypothesized that the weather conditions influenced the level and distribution of *Alternaria* mycotoxin contamination.

Little is known about the carryover of the *Alternaria* toxins into milled and cooked products or the long-term effects of low levels in animal diets. Further studies are clearly needed on the causes of the high levels of *Alternaria* infection in Argentinean wheat and what the long-term consequences are for the grain industries.

ACKNOWLEDGMENTS

Financial support of Universidad de Buenos Aires is acknowledged. We acknowledge Peter M. Scott for a critical reading of the manuscript and valuable suggestions.

REFERENCES

1. An, Y. H., T. Z. Zhao, J. Miao, G. T. Liu, Y. Z. Zheng, Y. M. Xu, and R. L. Van Etten. 1989. Isolation, identification and mutagenicity of alternariol monomethyl ether. *J. Agric. Food Chem.* 37:1341–1343.
2. Ansari, A. A., and A. K. Shrivastava. 1990. Natural occurrence of *Alternaria* mycotoxins in sorghum and ragi from North Bihar, India. *Food Addit. Contam.* 7:815–820.
3. Argentine Wheat. 2005. Institutional quality report. Available at: http://www.trigoargentino.com.ar. Accessed 19 September 2007.
4. Azcarate, M. P., G. Vaamonde, and V. Fernández Pinto. 2006. Toxinas de *Fusarium* y *Alternaria* en trigo cultivado en la provincia de La Pampa y sudeste de Buenos Aires, Argentina. 5th Latin American Congress of Mycotoxicology, 18 to 21 June 2006, Florianópolis, Brazil.
5. Bottalico, A., and A. Logrieco. 1998. Toxicogenic *Alternaria* species of economic importance, p. 65–108. *In* K. Kaushal and D. Bathnagar (ed.), Mycotoxins in agriculture and food safety. Marcel Dekker, Inc., New York.
6. Bruce, V. R., M. E. Stack, and P. B. Mislivec. 1984. Incidence of toxic *Alternaria* species in small grains from the U.S.A. *J. Food Sci.* 49:1626–1627.
7. da Motta, S., and L. M. Valente Soares. 2000. A method for the determination of two *Alternaria* toxins, alternariol and alternariol monomethyl ether, in tomato products. *Braz. J. Microbiol.* 31:315–320.
8. González, H. H. L., E. J. Martínez, A. Pacin, and S. L. Resnik. 1999. Relationship between *Fusarium graminearum* and *Alternaria alternata* contamination and deoxynivalenol occurrence on Argentinean durum wheat. *Mycopathologia* 144:97–102.
9. González, H. H. L., A. Pacin, S. L. Resnik, and E. J. Martínez. 1996. Deoxynivalenol and contaminant mycoflora in freshly harvested Argentinean wheat in 1993. *Mycopathologia* 135:129–134.
10. Grabarkiewicz-Szczęsna, J., J. Chelkowski, and P. Zajkowski. 1989. Natural occurrence of *Alternaria* mycotoxins in the grain and chaff of cereals. *Mycotoxin Res.* 5:77–80.
11. Lacey, J., and N. Magan. 1991. Fungi in cereal grains: their occurrence and water and temperature relationships, p. 77–118. *In* J. Chelkowski (ed.), Cereal grain. Mycotoxins, fungi, and quality in drying and storage. Elsevier, Amsterdam.
12. Li, F., and T. Yoshizawa. 2000. *Alternaria* mycotoxins in weathered wheat from China. *J. Agric. Food Chem.* 48:2920–2924.
13. Liu, G. T., Y. Z. Qian, P. Zhang, W. H. Dong, Y. M. Qi, and H. T. Guo. 1992. Etiological role of *Alternaria alternata* in human esophageal cancer. *Chin. Med. J. Engl. Ed.* 105:394–400.
14. Magan, N., and E. Baxter. 1994. Environmental factors and tenuazonic acid production by *Alternaria* spp. isolated from sorghum, p. 1043–1046. *In* E. Highley, E. J. Wright, H. J. Banks, and B. R. Champ (ed.), Proceedings of the 6th International Conference on Stored Product Protection. Commonwealth Agricultural Bureau International, Wallingford, UK.
15. Ostry, V., J. Skarkova, J. Nedelnik, J. Ruprich, and H. Moravcova. 2005. Occurrence of *Alternaria* and *Fusarium* mycotoxins in winter wheat from domestic crop in the year 2003. *Mycotoxin Res.* 21:23–25.
16. Patriarca, A., M. P. Azcarate, L. Terminiello, and V. Fernández Pinto. 2007. Mycotoxin production by *Alternaria* strains isolated from Argentinean wheat. *Int. J. Food Microbiol.* 119:219–222.
17. Pitt, J. I., and A. D. Hocking. 1985. Fungi and food spoilage. Academic Press, Sydney, Australia.
18. Samson, R. A., E. S. Hoekstra, J. C. Frisvad, and O. Filtenborg (ed.). 2002. Introduction to food and airborne fungi, 6th ed., 2nd printing. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
19. Sanchis, V., A. Sanclemente, A. Usall, and I. Vinas. 1993. Incidence of mycotoxigenic *Alternaria alternata* and *Aspergillus flavus* in barley. *J. Food Prot.* 56:246–248.
20. Sauer, D. B., L. M. Seitz, R. Burroughs, H. E. Mohr, J. L. West, R. J. Milleret, and H. D. Anthony. 1978. Toxicity of *Alternaria* metabolites found in weathered sorghum grain at harvest. *J. Agric. Food Chem.* 26:1380–1383.
21. Scott, P. M. 2001. Analysis of agricultural commodities and foods for *Alternaria* mycotoxins. *J. AOAC Int.* 84:1809–1817.
22. Scott, P. M. 2004. Other mycotoxins, p. 406–440. *In* N. Magan and M. Olsen (ed.), Mycotoxins in food: detection and control. Woodhead Publishing Ltd., Cambridge.
23. Scott, P. M., and D. R. Stolz. 1980. Mutagens produced by *Alternaria alternata*. *Mutat. Res.* 78:33–40.
24. Suter, D. A., C. A. W. Jackson, W. L. Bryden, S. Andrews, N. F. Tobin, and S. Jay. 1984. *Alternaria* toxins: a potential problem in animal feeds. *Aust. Microbiologist* 5:142.
25. Visconti, A., and A. Sibilia. 1994. *Alternaria* toxins, p. 315–336. *In* J. D. Miller and H. L. Trenholm (ed.), Mycotoxins in grains, compounds, other than aflatoxins. Eagan Press, St. Paul, Minn.
26. Webley, D. J., K. L. Jackson, J. D. Mullins, A. D. Hocking, and J. I. Pitt. 1997. *Alternaria* toxins in weather-damaged wheat and sorghum in the 1995–1996 Australian harvest. *Aust. J. Agric. Res.* 48:1249–1255.
27. Yekeler, H., K. Bitmis, N. Özçelik, M. Z. Doymaz, and M. Çalta. 2001. Analysis of toxic effects of *Alternaria* toxins on esophagus of mice by light and electron microscopy. *Toxicol. Pathol.* 29:492–497.