Alternaria Mycotoxins in Weathered Wheat from China

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This is the first report of the natural occurrence of *Alternaria* mycotoxins in Chinese wheat. Wheat kernels were significantly invaded by *Alternaria* species, mostly *A. alternata*, with an average infection frequency of 87.3%. A total of 22 samples of weathered wheat kernels from the 1998 crop, representing three locations in the suburbs of Beijing, China, were examined for the presence of *Alternaria* mycotoxins by high-performance liquid chromatography. Alternariol (AOH) was detected in 20 of 22 samples ranging between 116 and 731 μg/kg (mean = 335 μg/kg) and alternariol methyl ether (AME) at a mean level of 443 μg/kg (range = 52−1426 μg/kg) in 21 samples. The presence of tenuazonic acid (TA), a major *Alternaria* toxin in terms of quantity, was detected in all samples analyzed at an average concentration of 2419 μg/kg with a maximum of 6432 μg/kg. All samples were free from altertoxin I and altenuene. Samples with high levels of AOH and AME also contain a high level of TA. There was significant linear regression of correlations between the levels of AOH over AME (r = 0.850) and total benzopyrone derivatives (AOH + AME) over TA (r = 0.796).

Keywords: *Alternaria toxins; alternariol; alternariol methyl ether; tenuazonic acid; weathered wheat; China*

INTRODUCTION

Fusarium-associated damage in wheat and barley is very serious in China and widely prevalent. Trichothecences, a class of toxic metabolites produced by *Fusarium* species, are distributed countrywide as natural contaminants in these commodities. A number of outbreaks of intoxication in humans and animals have been reported in association with the consumption of foods or feeds contaminated with these mycotoxins (Luo, 1992; Li et al., 1999). Members of the genus *Alternaria*, which includes both plant pathogenic and saprophytic species that may cause extensive spoilage of crops in the field or after harvest, are also the major fungi found on wheat and barley in China. The incidence of *Alternaria* species in small grains, especially in wheat grains, is higher in the high-incidence areas for human esophageal cancer than in the low-incidence areas (Zhang et al., 1996). Moreover, culture extracts of *A. alternata* isolated from grains harvested in these regions were mutogenic in various microbial and cell systems (Dong et al., 1987; Liu et al., 1991) and tumorigenic in rats (Liu et al., 1984).

Alternaria species produce several mycotoxins belonging to three different structural groups: (i) dibenzopyrone derivatives of alternariol (AOH), alternariol methyl ether (AME), and altenuene (ALT); (ii) the perylene derivatives altertoxins (ATX-I and II); and (iii) the tetramic acid derivative tenuazonic acid (TA) (Figure 1). These toxins showed cytotoxic activity to bacterial and mammalian cells and fetotoxicity and teratogenicity to mice and hamsters (Visconti and Sibilia, 1994). AME and AOH were mutagenic in microbial and mammalian cell systems (An et al., 1989; Liu et al., 1992; Scott and Stolz, 1980). *Alternaria* toxins have been found to be natural food contaminants in grains, sunflower seeds, and some visibly decayed fruits in many countries (Scott and Stolz, 1980; David et al., 1978; Verneal et al., 1984; Suter et al., 1984; Ansari and Shrivastava, 1990; Webley et al., 1997). Although only a limited number of papers described the incidence of *Alternaria* species in local agricultural crops in China, the natural occurrence of *Alternaria* mycotoxins has not been reported previously

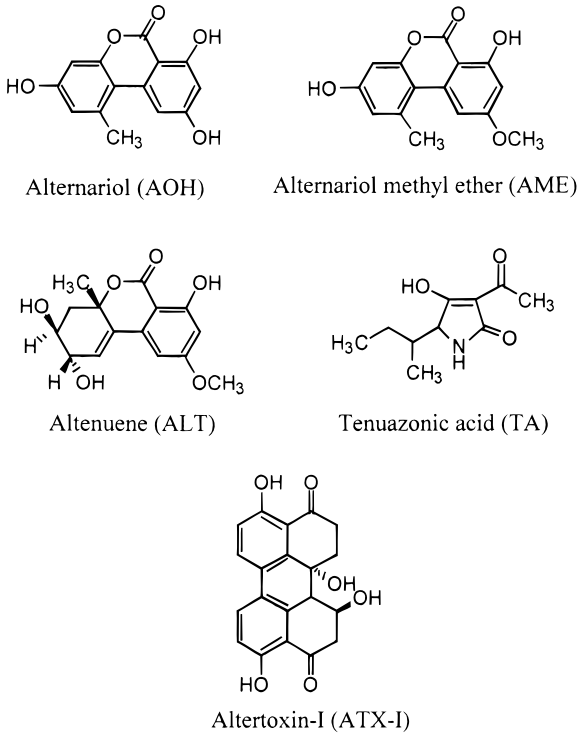

Figure 1. Structures of the investigated *Alternaria* mycotoxins.

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for Chinese agricultural commodities. The purpose of this study is to evaluate the incidence of *Alternaria* species and the natural occurrence of associated mycotoxins in 1998 weathered wheat from China. The results obtained from our study will be expected to contribute to ongoing research on the relationship between the consumption of cereals contaminated with *Alternaria* mycotoxins and the incidence of human esophageal cancer in China.

MATERIALS AND METHODS

Caution. *Alternaria* mycotoxins are known to have mammalian toxicity. Consequently, sample collection, fungal isolation, and solvent extracts should be handled with care. We followed Kagawa University Environmental Health Safety Guidelines for the use of dichloromethane.

Weathered Wheat Samples. Twenty-two wheat kernel samples (~250 g each) representing three geographical locations throughout the major wheat-producing area of Beijing suburbs (Miyun, Shunyi, and Tongxian Counties) were obtained by visiting the farmers' houses in July 1998 and maintained in zip-lock plastic bags at −30 °C before analysis. The local farmers typically stored the wheat grains in gunnysacks, wooden cribs, or cement jars. These samples were weather-damaged in the field due to excessive rainfall combined with high humidity and shortage of sunshine during the winter wheat cycle, especially late in the growing season. It was observed that in the heavily diseased kernels the whole grain was dark in color and had a gray-black discoloration with a black ring on the seed coat. The germ and beard ends in the mildly diseased kernels were covered by pale black spots. The percentages of dark discoloration of grains in the diseased fields, randomly checked by the government officer, were 10% for the lowest incidence, 40−50% for the moderate incidence, and >70% for the highest incidence.

Chemicals and Reagents. Standards for AOH, AME, ALT ATX-I, and TA (copper salt) were purchased from Sigma Chemical Co. (St. Louis, MO). All organic solvents were of reagent grade and distilled in glass apparatus before use.

Extraction of *Alternaria* Toxins. The extraction of *Alternaria* toxins from wheat samples was a modified procedure based on that of Visconti et al. (Visconti et al., 1986; Li and Yoshizawa, 1999). In brief, finely ground wheat samples (15 g) were extracted with acetonitrile/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 filtered again. The filtrate was divided into two parts. One part (75 mL) was extracted three times with 20 mL of dichloromethane. The organic phases were combined, evaporated to dryness, and dissolved in 1 mL of methanol for AOH, AME, ALT, and ATX-I analysis by HPLC. Another part (36 mL, equal to 2 g of sample) was adjusted to pH 2 with 6 N HCl, filtered again through cotton, and extracted twice for TA with 25 mL of dichloromethane. TA was then partitioned into 15 mL of 5% sodium bicarbonate, acidified to pH 2 again, and extracted twice with dichloromethane (15 mL). The dichloromethane extracts were combined, washed with 13 mL of water, and evaporated to dryness. The residue was made up to 2 mL with methanol and analyzed for TA by HPLC.

HPLC Determination. A Jasco PU-980 pump equipped with a Jasco AS-950 autosampler and either a Shimadzu RF-10AXL spectrofluorometer or a Shimadzu SPD-6A UV spectrophotometric detector was used. A short reverse phase column (TSK-gel ODS-80TM CTR, 5 μm particle size, 100 × 4.6 mm i.d., Tosoh, Tokyo, Japan) thermostated at 40 °C was employed for TA analysis. The chromatography of AOH, AME, ATX-I, and ALT was performed on a long reverse phase column (Shim-Pack CLC-ODS, 5 μm particle size, 250 × 4.6 mm i.d., Shimadzu Scientific Instrument Inc., Kyoto, Japan). Methanol/water (80:20, v/v) containing 300 mg of zinc sulfate/L was a mobile phase at a flow rate of 0.4 mL/min for AOH, AME, and ALT separation. Methanol/water in the ratios of 65:35 (v/v) and 85:15 (v/v) containing 300 mg of zinc sulfate/L at flow rates of 0.6 and 0.4 mL/min was the mobile phase for ATX-I and TA elution, respectively. A fluorescence detector with excitation/emission wavelengths of 253/415 nm was used for both AOH and AME; excitation/emission wavelengths of 243/460 nm were used for ALT analysis. The UV detector was set at 257 nm for ATX-I and at 280 nm for TA detection, with 0.01 absorbance unit full scale. Detection limits were 50 μg/kg for AOH and AME, 100 μg/kg for both ALT and TA, and 200 μg/kg for ATX-I.

Table 1. Occurrence of *Alternaria* Mycotoxins in Weathered Wheat from China[a]

	concentration, μg/kg			ratio of
	AOH	AME	TA	AOH/AME
no. of positive samples	20	21	22	
% positive	90.9	95.5	100.0	
range	116−731	52−1426	260−6432	0−2.28
average	335	443	2419	0.78

[a] Detection limits were 50 μg/kg for AOH and AME, 100 μg/kg for ALT and TA, and 200 μg/kg for ATX-I. ALT and ATX-I were not detected.

Mean recoveries of AOH, AME, ALT, and ATX-I from triplicate samples were 71, 96, 85, and 97% at 250 μg/kg with coefficients of variation (CV) of 2.44, 7.96, 8.35, and 1.57%, respectively, and 70, 96, 95, 94, and 56% at 500 μg/kg with CVs of 3.30, 2.08, 5.80, 3.74, and 2.53%, respectively. At 1000 μg/kg, the recoveries of ALT, ATX-I, and TA were 113, 96, and 64% with CVs of 0.51, 2.14, and 3.31%, respectively. The spiked level of TA at 2000 μg/kg gave a recovery of 71% (CV = 1.00%).

Confirmation was achieved by using a Shimadzu SPD-M10A VP photodiode array detector interfaced to an Shimadzu CBM-10A communication bus module and a Shimadzu Class-LC 10 model FMV-6300 DX 2c computer. The spectra were acquired in the range of 190−370 nm on the apex and on the ascending or descending part of each peak using a pilot signal at 257 nm for ATX-I, AOH, AME, and ALT and at 280 nm for TA. Reference spectra were acquired during the elution of associated standards and used for peak identification by comparison after spectral normalization. Additionally, thin-layer chromatography was also performed for the confirmation of the toxins by comparison of the spots from wheat extract with the corresponding standards as published previously (Visconti et al., 1986).

Isolation and Identification of Fungi. Wheat kernels were surface-disinfected with 1% sodium hypochloride for 2 min followed by rinsing with sterilized saline and incubated on potato dextrose agar media amended with 100 μg/mL chloramphenicol and maintained at 25 °C for 7 days. All colonies that developed from kernels were counted and identified in accordance with the literature (Ellis, 1971; Nelson et al., 1983; Barnett and Hunter, 1998).

RESULTS

Natural Occurrence of *Alternaria* Toxins in Chinese Weathered Wheat. Results of *Alternaria* toxin contamination in Chinese weathered wheat samples are given in Table 1. AOH and AME were detected in 20 (90.9%) samples at a level ranging from 106 to 731 μg/kg (mean = 335 μg/kg) and in 21 (95.5%) samples with a range of 52−1426 μg/kg (mean = 443 μg/kg), respectively. The ratio of AOH to AME ranged up to 2.28 (average = 0.82), among which 90.5% (19/21) were <1. Thus, the concentration of AOH was lower than that of AME in most of the samples analyzed. HPLC chromatograms for AOH and AME in a heavily contaminated wheat (sample BJT-98-W15) together with their standard toxins are shown in Figure 2.

TA was the predominant toxin quantitatively as shown in Table 1; it was detected significantly in all

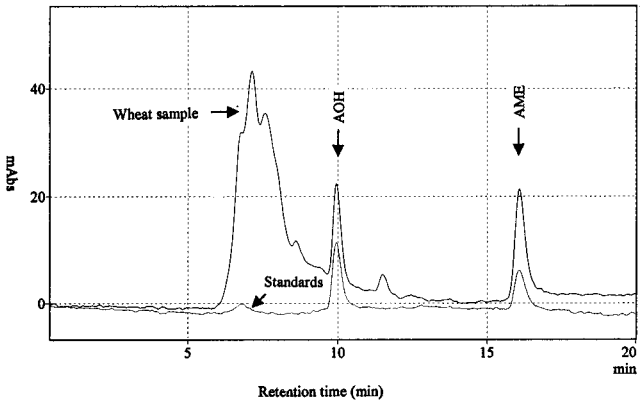

Figure 2. HPLC chromatograms of AOH and AME in a weathered wheat sample (BJT-98-W15) and associated standards.

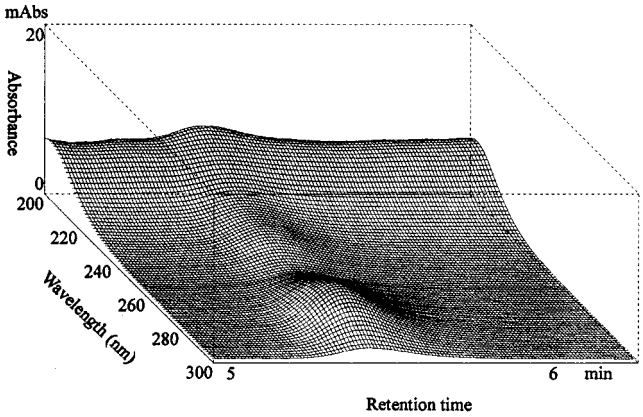

Figure 3. Three-dimensional spectrochromatogram for TA confirmation obtained from a wheat sample (BJT-98-W15).

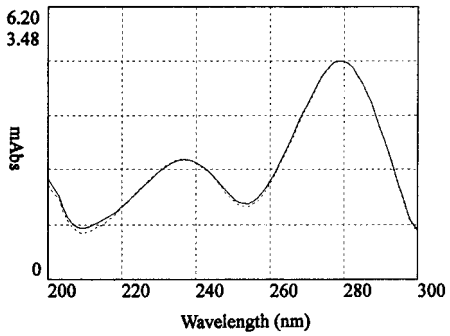

Figure 4. Spectrum of TA in a weathered wheat sample (BJT-98-W15) and its standard (dotted line) confirmed by photodiode array UV detector.

wheat samples at an average concentration of 2419 μg/kg. Three samples (13.6%) were contaminated with TA at >5000 μg/kg, with a maximum of 6432 μg/kg, 16 samples (72.7%) between 1000 and 5000 μg/kg, and only 3 samples (13.6%) at <1000 μg/kg. The presence of TA at a retention time of 5.44 min was confirmed by photodiode array detection as shown in Figures 3 and 4. Positive samples were also confirmed for AOH, AME, and TA by TLC. All of the samples analyzed were free from detectable amounts of ALT and ATX-I.

With regard to the co-occurrence of *Alternaria* toxins in weathered wheat, AME and TA occurred together in 21 samples (95.5%), whereas AOH, AME, and TA occurred in 20 samples (90.9%). A significant linear regression of correlation in concentrations of mycotoxins in weathered wheat was observed between AOH and

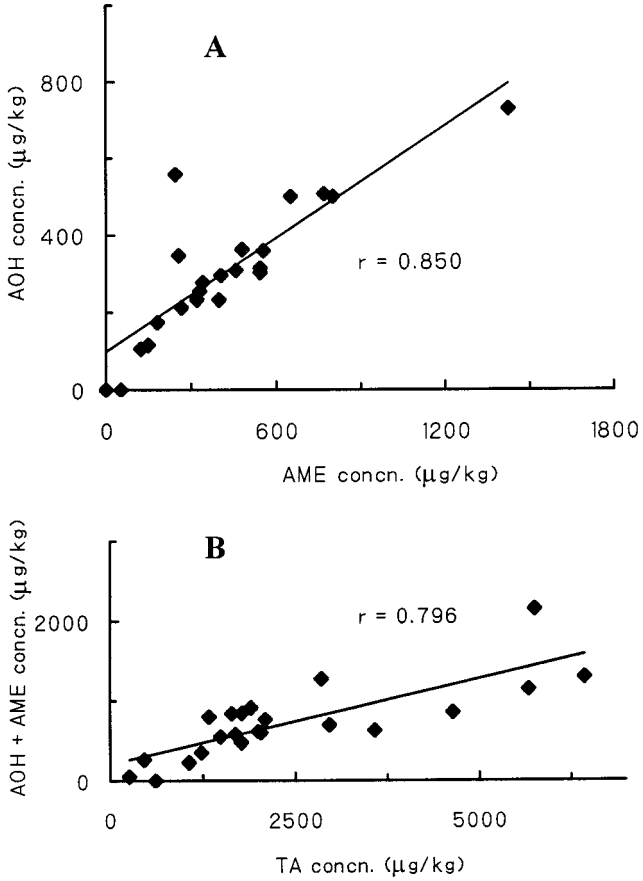

Figure 5. Correlations of concentration of *Alternaria* mycotoxins (AOH, AME, and TA) in Chinese weathered wheat: (A) AOH versus AME; (B) (AOH + AME) versus TA.

AME ($r = 0.850$, $p < 0.001$) and also the total dibenzopyrone derivatives (AOH + AME) and TA ($r = 0.796$, $p < 0.001$; Figure 5), indicating the coproduction of these toxins on wheat grains by *Alternaria* species in fields. 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 (Grabarkiewicz-Szczesna et al., 1989; Webley et al., 1997). The mycotoxigenicity of *A. alternata* isolates from these weathered wheat kernels will be published elsewhere.

Incidence of Mycoflora in Weathered Wheat Samples. Table 2 shows that all samples were invaded to varying degrees with various species of fungi. *Alternaria* species, identified mostly as *A. alternata*, were by far the predominant fungus and detected in all samples with an average percentage of kernel invasion of 87.3%. Wheat samples were also positive for species of *Drechslera*, *Penicillium*, *Fusarium*, *Aspergillus*, and others, including *Mocur*, *Rhizopus*, and some unidentified fungi with infection frequencies of 90.9, 54.6, 18.2, 13.6, and 63.6%, respectively, and average kernel invasions of 6.1, 2.3, 2.1, 8.9, and 6.7%, respectively.

The wheat cultivar (Jinghe No. 3) analyzed is new, with characteristics of large seed size and more tillering spikelets, but it is susceptible to fungal invasion. In 1998, the total daylight time and precipitation in the course of wheat earring, flowering, and maturing (early May to middle June) in the Beijing area were 85% and 147% of the normal year, respectively. In particular, the precipitation during the period of kernels at the milk stage (late May to early June) was 2 times higher than

Table 2. Incidence of Fungi Invasion in Weathered Wheat Kernels from China

			no. (%) invaded by					
	infected	germinated	*Alternaria*	*Fusarium*	*Aspergillus*	*Penicillium*	*Drechslera*	others[a]
no. of samples	22	22	22	4	3	12	20	14
%	100	100	100	18.2	13.6	54.5	90.9	63.6
% kernels								
range	84−100	6−46	60−96	2−2.9	4−17.2	1.8−4.7	1.8−15.5	1.8−37.1
average	97.3	19.9	87.3	2.1	8.9	2.3	6.1	6.7

[a] Others include *Mocur*, *Rhizopus* (constituted >80%), and unidentified fungi (<20%).

the normal. Therefore, the weather conditions of higher than normal humidity may have an important role in wheat invasion by *Alternaria* species.

Considering the phytotoxicity of *Alternaria* species on wheat grains tested, the incidence of seed germination on potato dextrose agar plates was estimated. It is worth noting that of 1100 wheat grains checked, only 219 (19.9%) germinated (Table 2). There appears to be a correlation between dark discoloration of the embryo end of wheat grains and lower germination rates.

DISCUSSION

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 (Visconti and Sibilia, 1994). The present paper provides additional information about the occurrence of AOH, AME, and TA in weathered wheat. To our knowledge, this is the first report of the natural occurrence of these mycotoxins in Chinese agricultural commodities. The characteristic distribution pattern of the toxins in the samples examined was observed: TA is a major toxin in all samples (average = 2419 μg/kg); AME was found at higher levels than AOH (Table 1). These results tended to be different from those reported in wheat from other countries. AOH was present as a major toxin in Australian weathered wheat, ranging in concentration between 10 and 1050 μg/kg; AME and TA were also detected at lower levels (Webley et al., 1997). Only AOH (590 μg/kg) was found in wheat kernels from Poland; the wheat was free from other *Alternaria* mycotoxins (Grabarkiewicz-Szczesna et al., 1989). Differences in geographical and environmental conditions might be responsible for these differences in mycotoxin distribution and concentrations. Moreover, TA levels, regardless of the average and maximum concentrations detected in Chinese wheat, were much higher than those in any other grains such as wheat, sorghum, and ragi reported previously (Grabarkiewicz-Szczesna et al., 1989; Ansari and Shrivastava, 1990; Webley et al., 1997). Contamination of Chinese wheat with such high levels of TA is a public health issue of concern because TA is the most important *Alternaria* mycotoxin in terms of its acute mammalian toxicity (Visconti and Sibilia, 1994).

Concerning the toxicity of AOH and AME, AME was highly mutagenic to *Escherichia coli* strain ND-160 without S9 activation (An et al., 1989). Both toxins bound to the DNA of esophageal epithelium of the human fetus, activated oncogenes in the human fetal epithelium, and induced the epithelial proliferation of human fetal esophagus in vitro (Liu et al., 1992). *Alternaria* species were most common mycoflora infecting main agricultural crops in areas with a high incidence of esophageal cancer in China, but the presence of mycotoxins produced by this genus has been largely ignored (Li et al., 1984; Liu et al., 1991; Zhang et al., 1996). Thus, the occurrence of these toxins in Chinese wheat is also an important issue in relation to the chronic health risk for esophageal cancer in humans.

Although wheat kernels analyzed were significantly invaded by *Alternaria* species as shown in Table 2, no significant difference in 1000 grain weight was observed between the wheat cultivar examined in this study and others ($p > 0.05$, data not shown). On the other hand, the frequency of invasion with *Fusarium* species, which generally led to lower weight of wheat kernels, was very low (2−2.9% kernels in only 4 of 22 samples, Table 2). This suggests that it is difficult to segregate damaged kernels from healthy ones, and the toxins may transfer into the flour once the weathered wheat is supplied in any extent for human consumption. Further studies are needed to clarify the possible transfer of the toxins into wheat flour after milling and their fates during food processing and cooking. Additionally, there is a need for more surveys on the natural occurrence of *Alternaria* toxins in wheat and related commodities to evaluate the extent of human exposure to *Alternaria* mycotoxins and the related toxicological importance in China.

ABBREVIATIONS USED

AOH, alternariol; AME, alternariol methyl ether; ALT, altenuene; ATX-I, altertoxin I; TA, tenuazonic acid; HPLC, high-performance liquid chromatography.

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