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Mycotoxin production by *Alternaria* strains isolated from Argentinean wheat

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Abstract

The toxigenic potential of *Alternaria* strains isolated from Argentinean wheat was investigated. A total of 123 strains were assayed for the production of tenuazonic acid (TA), alternariol (AOH) and alternariol monomethyl ether (AME). All but one of the isolates were able to produce at least one of the three mycotoxins. TA was produced by 72% of the strains (1–14782 mg/kg), AOH by 87% (4–622 mg/kg) and AME by 91% (7–2625 mg/kg). The average level of TA detected for all strains (1757 mg/kg) was higher than the average level of both alternariols (162 mg/kg for AOH and 620 mg/kg for AME). TA was the toxin produced at the highest concentration but in lower frequency. Most of the strains were able to synthesize more than one toxin: 74 isolates (60%) were positive for all three toxins, 30 (24%) for both AOH and AME, 5 (4%) for both TA and AME, and 2 (2%) for TA and AOH. The widespread occurrence of *Alternaria* in wheat and its ability to produce mycotoxins suggests the possible occurrence of its toxins in wheat naturally infected with this fungus.

Keywords: *Alternaria*; Mycotoxins; Wheat; Tenuazonic acid; Alternariol; Alternariol monomethyl ether

1. Introduction

Argentina is one of the world's largest wheat (*Triticum aestivum* L.) producing countries. It has a large cultivated area with yields of more than 15 million metric tons a year. Two thirds of this production is exported (Argentine Wheat, 2005). Wheat is the main cereal used for human consumption in the country (Gallo et al., 1992; García, 2005).

The genus *Alternaria* includes plant pathogenic and saprophytic species that may affect crops in the field or can cause harvest and postharvest decay of plant products. Some species can produce mycotoxins in infected plants and/or in agricultural commodities (Logrieco et al., 2003). Cereal grains are frequently infected by species of *Alternaria*, particularly *A. alternata* which can cause a disease called "black point" which consists of a discoloration of the germ and the seed due to mycelial and conidial masses. This disease is frequent and serious when persistent rainfall, heavy dews or irrigation occur during kernel development, although a high incidence has also been observed in relatively dry weather (Logrieco et al., 2003).

The major *Alternaria* mycotoxins belong to three structural classes: the tetramic acid derivative, tenuazonic acid (TA); the dibenzopyrone derivatives, alternariol (AOH), alternariol monomethyl ether (AME) and altenuene; and the perylene derivatives, the altertoxins (Bottalico and Logrieco, 1998). The toxicity of TA has been reported in plants, in chick embryos and several other animal species, including guinea pigs, mice, rabbits, dogs, and rhesus monkeys (Visconti et al., 1987; Visconti and Sibilia, 1994). AOH and AME are mutagenic and cytotoxic to bacterial and mammalian cells, and are suspected to be carcinogenic (Pero et al., 1973; Harvan and Pero, 1976; Woody and Chu, 1992; Visconti and Sibilia, 1994; Scott, 2001). Both AOH and AME cause weakly acute toxicity but show synergistic effects (da Motta and Valente Soares, 2000). Consumption of cereals invaded by *Alternaria* and contaminated with associated mycotoxins was related to risk of human esophageal cancer in China (Liu et al., 1992). Although *Alternaria* species are very commonly found on

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grain, the presence of *Alternaria* mycotoxins has been largely ignored in these products (Webley et al., 1997). There are no specific international regulations for any of the *Alternaria* toxins in food (Scott, 2004).

The objective of the present study was to determine the toxigenic capacity of *Alternaria* spp. isolated from wheat grown in Argentina, in order to evaluate a potential risk to human and animal health.

2. Materials and methods

2.1. Wheat samples

A total of 41 samples of wheat from the Argentinean wheat production area known as V-South (La Pampa and SW Buenos Aires provinces) were used in this study. Samples were at least 1 kg in size and were collected arbitrarily from farms in the region during the 2004–2005 harvest (December 2004 through January 2005). The climate of region V-South was wetter than usual during the harvest period (Argentine Wheat, 2005). The kernels were harvested at maturity with 14% of moisture. All samples were from locally grown cultivars and not sorted by quality (all grades included). Sample processing was performed at "Chacra Experimental Integrada Barrow" and Estación Experimental Agropecuaria Bordenave (Instituto Nacional de Tecnología Agropecuaria — INTA).

2.2. Isolation and identification of fungi

For the isolation of the internal fungi, 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 in a sterile laminar flow cabinet. A total of 100 kernels per subsample were placed, 10 per plate, on potato dextrose agar (PDA) and incubated for 5 days at 25 °C under fluorescent light (12 h photoperiod). The resulting fungal colonies were enumerated and the genera were identified according to Pitt and Hocking (1997). *Alternaria* colonies were selected at random from each subsample and subcultured in dichloran chloramphenicol malt extract agar (DCMA, Andrews et al., 1992) for identification according to Simmons (1993, 1999) and Simmons and Roberts (1993). A total of 123 *Alternaria* spp. single-spore isolates were made and identified from the 41 wheat samples used in this study.

2.3. Mycotoxin production

Flasks were made up containing 12.5 g of autoclaved polished rice at 40% moisture. Flasks were inoculated with agar plugs of one-week-old cultures of *Alternaria* spp. isolate. The flasks were incubated in the dark at 25 °C for 21 days (Li et al., 2001).

2.4. Extraction of Alternaria toxins

The method for the detection of *Alternaria* toxins in rice was described by Li et al. (2001). The culture material was homogenized with 30 ml of methanol and filtered through a Whatman filter paper (no. 1). The filtrate was clarified with 60 ml of 20% ammonium sulphate and divided into two parts. One part (40 ml) was extracted three times with 10 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). Another part, (20 ml) was adjusted to pH 2 with 6 N HCl and extracted twice for TA with 15 ml of chloroform. TA was then partitioned into 10 ml of 5% sodium bicarbonate, acidified to pH 2 again, and extracted twice with 10 ml of chloroform. The chloroform extracts were combined, washed with 7.5 ml of water, and evaporated to dryness. The residue was made up to 4 ml with methanol and analyzed for TA by HPLC.

2.5. HPLC detection

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 4.6×250 mm 5 µ C18 (Phenomenex, USA). Standards of TA, AME and AOH were purchased from SIGMA Chemical Company (St. Louis, MO, USA). The mobile phase was methanol/water (80:20) containing 300 mg $ZnSO_4 \cdot H_2O$/l, for AOH and AME, and methanol/water (85:15) containing 300 mg $ZnSO_4 \cdot H_2O$/l, 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. The spectra were acquired in the range of 200–300 nm. 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 11 µg/kg for TA, 2 µg/kg for AME, and 5 µg/kg for AOH.

3. Results and discussion

3.1. Distribution of Alternaria species

The genus *Alternaria* was the main component of the wheat mycota. It was present in 100% of samples analyzed with an average kernel infection rate of 85%. This incidence is similar to the results of Webley et al. (1997), González, Martinez, Pacin, and Resnik (1999), and Li and Yoshizawa (2000). *Fusarium* spp. also showed a high infection rate in kernels, but was found in only 2% of samples. Other genera isolated with a relatively high frequency were *Epicoccum*, *Chaetomium* and *Sordaria*.

The frequency of *Alternaria* spp. isolated from Argentinean wheat is shown in Table 1. González, Pacin, Resnik, and Martinez (1996), and González et al. (1999) reported the same two predominant species in Argentinean common and durum wheat, although they found *A. alternata* as the most frequent species. Kosiak et al. (2004) found that *A. infectoria* was the most common species group in Norwegian grains followed by *A. tenuissima*. *A. alternata* was the most frequent species