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Two-dimensional environmental profiles of growth and fumonisin production by *Fusarium proliferatum* on a wheat-based substrate

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ABSTRACT

The effect of water activity (a_W; 0.995, 0.99, 0.98, 0.96, 0.94, 0.92, and 0.90), temperature (15, 25, and 30 °C), incubation time (7, 14, 21 and 28 days), and their interactions on mycelial growth and fumonisin production on wheat-based medium by three *Fusarium proliferatum* strains isolated from wheat in Argentina was evaluated. Maximum growth rates were obtained at the highest a_W (0.995) and 30 °C, with growth decreasing as the a_W of the medium was reduced. Maximum amounts of total fumonisins (FB_1, FB_2 and FB_3) were produced at 0.99 a_W and 25 °C after 21 and 28 days of incubation for 2 strains, and at 15 °C and 0.98 a_W after 28 days of incubation for the third strain. The fumonisin concentrations varied considerably depending on the a_W and temperature interactions assayed. The studied strains had different fumonisin production profiles. *F. proliferatum* ITEM 15661 and ITEM 15664 produced FB_1 and FB_2 whereas *F. proliferatum* ITEM 15654 was able to produce FB_1, FB_2 and FB_3. Interestingly, fumonisin production profiles for each particular strain were related to incubation temperatures. Fumonisins were produced from 15 to 30 °C and at a_W values of 0.92 to 0.995 after 21 to 28 days of incubation. However at 7 and 14 days of incubation small amounts of fumonisin were produced at a_W lower than 0.94. Two-dimensional profiles of a_W by temperature interactions were developed from these data to identify areas where conditions indicate a significant risk from fumonisin accumulation on wheat. Temperature and a_W conditions that resulted in fumonisin production are those found during wheat grain development (especially milk and dough stages) in the field. This study provides useful base line data on conditions representing a high and a low risk for contamination of wheat by fumonisins which is becoming of greater concern because this cereal is destined mainly for human consumption.

1. Introduction

Wheat is the most important cereal consumed by the Argentinean population. In this country human consumption of wheat manufactured products, either semolina (*Triticum turgidum* L. var. durum) or bread (*Triticum aestivum*), is much greater than for products made from other cereals (Food Balance Sheet, 2007; Pacin et al., 2012). In Argentina durum wheat is mainly used for pasta manufacture, with production reaching 604,651 tons in 2011. Pasta production reached almost 183,000 tons in 2011, and the consumption per capita was estimated at 7.9 kg/year. Common wheat production was of 15,271,000 tons in 2012 (MAGyP, 2014). It is used mostly for manufacture of bread, breakfast cereals, cookies and cupcakes. It is remarkable that wheat flour consumption in Argentina was estimated at 7.4 kg/person/month.

Fusarium species can produce a range of mycotoxins which endanger the health of both humans and animals (Sumalan et al., 2013). The main pathogen associated with *Fusarium* head blight (FHB) in common and durum wheat in Argentina is *Fusarium graminearum* sensu stricto (Lori et al., 2003; Ramirez et al., 2006b, 2007). Deoxynivalenol (DON) contamination has been reported in both wheat types (Dalcero et al., 1997; González et al., 1997; Lori et al., 2003). However Ramirez et al. (2006a) and Palacios et al. (2011) carried out two mycological surveys during non-FHB epidemic years in common wheat and durum wheat, and found that the predominant *Fusarium* species was *Fusarium proliferatum*. Natural fumonisin contamination (mainly fumonisin B_1) was reported for the first time on durum (Palacios et al., 2011) and common wheat (Cendoya et al., 2014) in Argentina during FHB non-epidemic years. The high frequency of fumonisin contamination found (more than 90%) in both kinds of wheat and also the fact that some samples evaluated in both studies exceeded the limits established for maize and sub-products for human consumption, which is 1000 ng/g in the European Union, are important to note. The occurrence of fumonisin on wheat is of concern, since the consumption of fumonisin-contaminated maize has been epidemiologically associated with esophageal cancer (Marasas, 2001) and neural tube defects in some human populations (Missmer et al., 2006). Consequently, the International

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Agency for Research on Cancer (IARC) designated FB_1 in Group 2B as "a possible carcinogenic to humans" (IARC, 2002).

Due to the importance of wheat in the Argentinean diet, and because it has been proposed, in a study in the Netherlands, that fumonisin intake occurs mainly via the intake of wheat and wheat-products (Bakker et al., 2003), it is relevant to understand the ecology of this species on wheat.

It is well known that fungal growth and mycotoxin production result from the complex interaction of several factors and, therefore, an understanding of each factor involved is essential to understand the overall process and to predict and prevent mycotoxin development (Chamley et al., 1994). Temperature and water activity (a_W) are the primary environmental factors that influence growth and mycotoxin production by fungi, including *Fusarium* species (Marín et al., 2004). While information is available on the relationship between these factors and profiles for growth and DON production by *F. graminearum* on wheat (Ramirez et al., 2006b), there is no practical information for *F. proliferatum* on this substrate.

The aim of this work was to determine the impact of a_W, temperature, and incubation time on growth and fumonisin production on wheat extract agar by three strains of *F. proliferatum* isolated from wheat in Argentina.

2. Material and methods

2.1. Fungal strains

Three *F. proliferatum* strains ITEM 15654, ITEM 15661 and ITEM 15664 (ITEM: Agri-Food Toxigenic Fungi Culture Collection of the Institute of Sciences of Food Production, CNR, Bari, Italy; http://www.ispa.cnr.it/Collection) previously isolated from wheat grains in Argentina during 2007–2008 harvest season were used. These isolates have been characterized by molecular, biological and morphological criteria (Nelson et al., 1983; Leslie and Summerell, 2006). For the molecular characterization, sequences of elongation factor (*EF-1α*), *calmodulin*, and *FUM 8* genes indicated that these isolates belong to the *Gibberella fujikuroi* species complex and were characterized as *F. proliferatum*. In order to determinate their *G. fujikuroi* mating population (MP) crossing experiments were performed (Klittich and Leslie, 1998) with standard testers as female parents and the uncharacterized field isolates as male parents. All these strains belong to *G. fujikuroi* mating population D and their mating types are MATD-1. Also, all isolates produce fumonisins (Palacios et al., 2011). Cultures were maintained in 15% glycerol at −80 °C.

2.2. Medium

Wheat, free of fumonisin contamination, was finely milled by using a Romer mill (Romer Labs Inc., Union, MO, USA). Mixtures of 2% (w/v) of milled wheat in water were prepared and 2% (w/v) agar (technical agar N° 2, Oxoid) added. The a_W of the basic medium was adjusted to 0.995, 0.99, 0.98, 0.96, 0.94, 0.92, and 0.90 by addition of different amounts of glycerol (Dallyn and Fox, 1980). The media were autoclaved at 120 °C for 20 min. Flasks of molten media were thoroughly shaken, prior to pouring into 9 cm sterile Petri dishes. The water activity of representative samples (2 of each treatment) of media was checked with an Aqualab Series 3 (Decagon Devices, Inc., WA, USA). Additional, uninoculated control plates were prepared and measured at the end of the experiment in order to detect any significant deviation of the a_W.

2.3. Inoculation, incubation, and growth assessment

Petri plates were inoculated with a 4-mm-diameter agar disk that was taken from the margin of a 7-day-old colony of each isolate grown on synthetic nutrient agar (Gerlach, and Nirenberg, 1982) at 25 °C and transferred face down to the center of each plate. Inoculated plates of the same a_W were sealed in polyethylene bags and incubated at 15, 25, and 30 °C for 28 days. A full factorial design was used where the factors were a_W, temperature and strain, and the response was growth (total number of plates: 7 a_W × 3 temperatures × 3 strains × 3 replicates).

Assessment of growth was made every day during the incubation period, and two diameters of the growing colonies were measured at right angles to each other until the colony reached the edge of the plate. Colonies radii were plotted against time, and linear regression was applied in order to obtain the growth rate (mm/day) as the slope of the line. After the incubation period, uninoculated controls and treatments were frozen for later extraction and fumonisin determination.

2.4. Determination of fumonisins

For fumonisin extraction Petri plates of each strain at different incubation periods (7, 14, 21 and 28 days) and for every a_W and temperature condition were used. Toxins were extracted with acetonitrile: water (1 : 1 v/v) by shaking the whole culture media (~20 g) and mycelia with the solvent for 30 min on an orbital shaker (150 rpm) and then filtering the extracts through filter paper (No. 4; Whatman International Ltd., Maidstone, Kent, UK). An aliquot of the extracts (1000 μL) was taken and diluted with acetonitrile: water (1: 1 v/v) as necessary for high performance liquid chromatography (HPLC) analysis. An aliquot (50 μL) of this solution was derivatized with 200 μL of an o-phthaldialdehyde (OPA) solution obtained by adding 5 mL of 0.1 M sodium tetraborate and 50 μL of 2-mercaptoethanol to 1 mL of methanol containing 40 mg of OPA (Shephard et al.,1990). The fumonisin OPA derivates (50 μL solution) were analyzed by using reversed-phase HPLC/fluorescence detection system. The HPLC system consisted of a Hewlett-Packard 1100 pump (Hewlett-Packard, Palo Alto, CA, USA) connected to a Hewlett-Packard 1046A programmable fluorescence detector and a data module Hewlett-Packard Kayak XA (HP ChemStation Rev. A.06.01). Chromatographic separations were performed on a stainless steel, C_{18} reversed-phase column (150 × 4.6 mm i.d., 5 μm particle size; Luna-Phenomenex, Torrance, CA, USA) connected to Security Guard cartridge (4 × 3 mm i.d., 5 μm particle size; Phenomenex, Torrance, CA, USA) filled with the same phase. Methanol:0.1 M sodium dihydrogen phosphate (75:25, v/v) solution adjusted to pH 3.35 with orthophosphoric acid was used as the mobile phase, at a flow rate of 1.5 mL/min. Fluorescence of the fumonisin OPA derivatives was recorded at excitation and emission wavelengths of 335 and 440 nm, respectively. Fumonisins were measured as peak heights and compared with reference standard solutions of fumonisins B_1, B_2 and B_3 (Sigma Chemical Co., St. Louis, MO, USA). A mixed acetonitrile:water (1:1, v/v) stock solution of FB_1, FB_2 and FB_3 containing 50 μg/mL of each toxin was prepared. Four mixed working calibrant solutions (0.25, 0.5, 1.0, and 2.0 μg/mL) were prepared by diluting an aliquot of the stock solution with the appropriate volume of acetonitrile:water (1:1, v/v). The retention time of FB_1, FB_3 and FB_2 was 7.5, 16.7 and 18.5 min, respectively. Appropriate dilutions of standards and/or sample extracts were made with acetonitrile/water (1:1). The detection limit of the analytical method for the three fumonisins was 1 μg/g based on the signal-to-noise ratio 3:1. Recovery experiment was performed on 2% milled maize agar spiked at levels of 1 to 10 μg/g of each fumonisin (FB_1, FB_2 and FB_3). Mean recovery ranged from 95 to 98% and 94% for FB_1, FB_2 and FB_3, respectively.

2.5. Statistical analysis

The growth rates and mycotoxin concentration were evaluated by analysis of variance (ANOVA) to determine the effect of a_W, temperature and *F. proliferatum* strains and two- and three-way interactions. When the analysis was statistically significant, the post hoc Tukey's multiple comparison procedure was used for separation of the means. Statistical significance was judged at the level $P \leq 0.01$. Statistical analysis was done using SigmaStat for Windows Version 2.03 (SPSS Inc.).