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Fungal screening and aflatoxin production by *Aspergillus* section *Flavi* isolated from pre-harvest maize ears grown in two Argentine regions

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ABSTRACT

Aflatoxin contamination in maize kernels grown in central Argentina has increased since 2008. Pre-harvest maize ear samples were collected during two growing seasons to determine prevalent fungal genera as well as aflatoxin production capacity by *Aspergillus* sect. *Flavi* in two maize regions. A direct planting procedure was performed for fungal screening. *Aspergillus* section *Flavi* was identified via a morphological analysis and some colonies were isolated. Strains were subjected to molecular analysis and aflatoxin production was studied. *Fusarium* was the fungus genus with the highest severity, followed by *Penicillium* and *Aspergillus* in both regions. *Aspergillus* sect. *flavi* were detected in 27 (73%) samples. PCR amplifications were observed in 94% of the isolates. *Aspergillus* sect. *flavi* severity varied significantly between years. A great proportion of toxigenic strains were observed in a native *Aspergillus* sect. *flavi* population (95%) producing B- and G-type aflatoxins. Maize ears grown in regions I and IV in Argentina are prone to degradation by fungi. Toxigenic strains are predominant in native *Aspergillus* sect. *Flavi* populations. Aflatoxin contamination of maize kernels is not restricted to post-harvest; rather, it is a serious issue that begins with colonization by *Aspergillus* sect. *Flavi* in ears.

1. Introduction

Maize (*Zea mays* L.) is widely grown in Argentina in nine regions (I to IX) under different agro-ecological conditions (INTA, 1997). In regions I and IV, the crop production has greatly increased due to expansion of the agricultural frontier and the adoption of technology (Viglizzo et al., 2012).

Environmental conditions influence the fungal colonization of maize ears during flowering (Diener, 1989; Perrone et al., 2014b). This period usually spans December to March in Argentina, and varies among planting regions (Cirilo, 2004). Fungi may affect maize directly by mechanical damage causing yield losses, or indirectly, by secreting and spreading mycotoxins (Iheanacho et al., 2014). *Fusarium* Link. is the most important pathogenic fungal genus causing spoilage of maize, with *F. verticillioides* (Sacc.) and *F. graminearum* Schwabe Nirenberg being the most common species frequently isolated from maize kernels (Etcheverry et al., 1999; Presello and Botta, 2004). *Penicillium* Link species are also present in maize ears and produce a wide range of secondary metabolites, including ochratoxin, citrinin and scalonic acid D (Pitt and Hocking, 2009). Aflatoxins are secondary metabolites produced in nature by several *Aspergillus* Fr.:Fr. species. *A. flavus* Link and *A. parasiticus* Speare are the most common species associated with aflatoxin contamination in crops (Perrone et al., 2014a). Recently, other species of *Aspergillus* section *Flavi* were reported as responsible for aflatoxin contamination in maize (Perrone et al., 2014b; Soares et al., 2012). Previous studies in Argentina indicated the presence of *Aspergillus* sect. *Flavi* in stored maize grains as well as in soil, debris and insects from maize fields (Nesci and Etcheverry, 2002; Nesci et al., 2006).

Maize regions I and IV are characterized by extreme temperatures and hot dry weather during the maize growing period (SMN,

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2015). The *Aspergillus* sect. *Flavi* population increases in crops grown under these climate conditions (Bhatnagar-Mathur et al., 2015). High levels of aflatoxins in maize grown in regions I and IV have been reported by the Argentinian maize industry. More than 30% of the crop exceeds the limit of 20 parts per billion of total aflatoxins established by international regulations (personal communication, June 9, 2011).

Some *Aspergillus* species are similar and identification to species level using schemes based on morphological characteristics requires expertise. Effective management of disease caused by *Aspergillus* species requires proper identification of the fungal pathogen (Gherbawy and Voigt, 2010). Molecular identification of fungi was reported to be a reliable method to confirm the identity of strains previously identified using the conventional method based on morphological characteristics (Iheanacho et al., 2014). Devi et al. (2013) developed a marker for accurately differentiating *A. flavus* isolates from other *Aspergillus* species by polymerase chain reaction (PCR). This primer pair has not been tested in strains obtained from Argentine crops.

Populations of *Aspergillus* sect. *Flavi* exhibit extremely diverse toxigenic characteristics. *A. flavus* produces B-type aflatoxins (B_1 and B_2) and often cyclopiazonic acid (CPA) depending on the geographic origin, substrate, genotype and environmental conditions. *A. parasiticus* produces G-type aflatoxins (G_1 and G_2) in addition to B-type aflatoxins (Perrone et al., 2014b; Vaamonde et al., 2003). Recently, new aflatoxigenic species that share morphological characters with *A. flavus* were reported to produce both B-type and G-type aflatoxin but the exact taxonomic designations remain unclear (Donner et al., 2009; Perrone et al., 2014b). Vaamonde et al. (2003) proposed classifying *Aspergillus* sect. *Flavi* isolated from several Argentine crops into groups according to the production of B- and G-type aflatoxins. All aflatoxins are teratogenic and carcinogenic, with Aflatoxin B1 being the metabolite with the highest toxigenic effect on humans and animals (Peraica et al., 1999).

An appropriate disease management strategy to reduce the presence of mycotoxins in maize crops requires knowledge about the incidence and toxicity of *A. flavus* strains present in ears. Kernels infected during the growing season remain as inoculum. Under improper storage conditions, fungi grow and aflatoxin contamination increases during the post-harvest period (Bhatnagar-Mathur et al., 2015; Williams et al., 2011). In order to develop disease management strategies for maize regions I and IV in Argentina, the aims of this work were to: study the severity of the main toxigenic genera (*Fusarium*, *Penicillium* and *Aspergillus*) present in corn ears; record the severity of *Aspergillus* sect. *Flavi* identified using morphological criteria; molecularly characterize native *Aspergillus* sect. *Flavi* strains by PCR; estimate the percentage of toxigenic *Aspergillus* sect. *Flavi* strains, and quantify their aflatoxin production.

2. Materials and methods

2.1. Sampling

Samples of different commercial maize cultivars were collected from the maize regions I and IV in Argentina during the 2012/13 and 2013/14 growing seasons (Fig. 1) (INTA, 1997). Region I lies approximately between latitudes 20°00′ and 30°00′ S, with average maximum temperatures varying from 30 to 34 °C during the maize flowering period. Region IV is located approximately between latitudes 30°00′ and 34°00′S, with average maximum temperatures ranging from 26 to 30 °C during the same period. Both regions are located between longitudes 62°00′and 67°00′W, where rainfall is between 200 and 500 mm during the flowering period (SMN, 2015). Weather conditions varied remarkably between the growing seasons studied (Table 1) (INTA, 2016). Ten ears were randomly selected from fields at the end of the growing season, immediately before harvest. Samples were transported in paper bags and dried in a forced air oven (38 °C for 72 h) to reduce moisture content to below 12%. Samples were stored at 4 °C until analysis (Oliveira Rocha et al., 2012; Smart et al., 1990).

2.2. Fungal screening

A direct planting procedure for internal infestation was carried out to determine the main fungal genera present in maize ears (Pitt and Hocking, 2009). Sample grains (200 grains/sample), obtained by threshing of ears, were surface-disinfected by dipping in 1% sodium hypochlorite solution for 5 min and rinsed three times in sterile water. Each sample was analyzed in two culture media, Dichloran Rose-Bengal Chloramphenicol Agar (DRBC) and Dichloran Glycerol Agar (DG18) (Merck, Darmstadt, Germany), using 10 Petri dishes per medium. Ten grains per dish were planted directly on the medium surface and incubated at 25 ± 2 °C in darkness for 7 days (Giorni et al., 2007). After the growing period, samples were examined and the number of grains with fungus colonies exhibiting morphological characteristics consistent with those of *Aspergillus*, *Penicillium*, *Fusarium* and other fungus species was counted (Perrone et al., 2014b). Results are expressed as severity (percentage of infected grains) and incidence (percentage of infected samples).

2.3. Morphological identification of Aspergillus sect. Flavi

Spores from colonies that looked like *Aspergillus* sect. *Flavi* were transferred and sub-cultured on malt extract agar medium (MEA) at 25 °C in darkness during 7 days for further identification (Nesci and Etcheverry, 2002). *Aspergillus* sect. *Flavi* species were determined by observing colony characteristics and conidial morphology, following taxonomic schemes proposed by Pitt and Hocking (2009). The presence of species of *Aspergillus* sect. *Flavi* was expressed as severity and incidence. Colonies were isolated for further molecular characterization and determination of aflatoxin production.

2.4. Molecular characterization of Aspergillus sect. Flavi isolates

2.4.1. Fungal DNA extraction

Culture medium (20% potato leachate; 2% sucrose; pH 4.5) was prepared and transferred (100 mL) to 250-mL flasks. Spore suspensions were prepared from previously grown *Aspergillus* sect. *Flavi* isolates (at 25 °C in darkness for 7 days) and added to the medium (1×10^5 conidia/mL). Flasks were placed on a shaker at 250 rpm and incubated at 25 °C for 48 h. The mycelia were harvested by filtration through Whatman N° 1 filter, rinsed three times with sterile water and stored lyophilized at −40 °C (Devi et al., 2013). DNA was extracted following the procedure described by Doyle and Doyle (1990). *A. niger* isolated from maize kernels was used as a negative control. *A. flavus* 114116 provided by the Instituto Nacional de Enfermedades Infecciosas (Buenos Aires, Argentina) was used as a positive control. *A. parasiticus* NRRL 2999 provided by Universidad Nacional de Rosario (Santa Fe, Argentina) was also tested as an additional control in order to determine if the primer studied is suitable for distinguishing this species from *A. flavus*.

2.4.2. Primers and PCR conditions

The primers used in the reaction had the following nucleotide sequence: Asp f1, 5′-CCCGTGAAGTTGCCCAGGT-3'; Asp r2, 5′-GTCGTTTGGTGAGTGGGAA-3′ (Devi et al., 2013). Reagents were