International Journal of Food Microbiology 277 (2018) 58–63

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Characterization and competitive ability of non-aflatoxigenic *Aspergillus flavus* isolated from the maize agro-ecosystem in Argentina as potential aflatoxin biocontrol agents

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ARTICLE INFO

Keywords:
Biocontrol
Maize
Aspergillus flavus
Aflatoxins
Cyclopiazonic acid

ABSTRACT

Aspergillus flavus is an opportunistic pathogen and may produce aflatoxins in maize, one of the most important crops in Argentina. A promising strategy to reduce aflatoxin accumulation is the biological control based on competitive exclusion. In order to select potential biocontrol agents among isolates from the maize growing region in Argentina, a total of 512 *A. flavus* strains were isolated from maize kernels and soil samples. Thirty-six per cent of the isolates from maize kernels did not produce detectable levels of aflatoxins, while 73% of the isolates from soil were characterized as non-aflatoxin producers. Forty percent and 49% of the isolates from maize kernels and soil samples, respectively, were not producers of cyclopiazonic acid (CPA). Sclerotia morphology was evaluated using Czapek Dox media. Eighty-six per cent of the isolates from maize kernels and 85% of the isolates from soil samples were L sclerotia morphotype (average diameter > 400 μm). The remaining isolates did not produce sclerotia. All isolates had *MAT 1-1* idiomorph. The competitive ability of 9 non aflatoxigenic strains, 4 CPA(+) and 5 CPA(−), was evaluated in co-inoculations of maize kernels with an aflatoxigenic strain. All evaluated strains significantly ($p < 0.05$) reduced aflatoxin contamination in maize kernels. The aflatoxin B_1 (AFB_1) reduction ranged from 6 to 60%. The strain *A. flavus* ARG5/30 isolated from maize kernels would be a good candidate as a potential biocontrol agent to be used in maize, since it was characterized as neither aflatoxin nor CPA producer, morphotype L, *MAT 1-1* idiomorph, and reduced AFB_1 content in maize kernels by 59%. This study showed the competitive ability of potential aflatoxin biocontrol agents to be evaluated under field trials in a maize agro-ecosystem in Argentina.

1. Introduction

Maize (*Zea mays* L.) is one of the most important crops in Argentina, principally in Buenos Aires, Córdoba and Santa Fe provinces. The country occupies the 5th position as leading maize producer and the 2nd position in the world as maize exporter (BCP, 2016; USDA, 2017). Since 2006/07 growing season, an increase in total maize planted area and consequently in production was observed in Argentina (MAGyP, 2017).

Among the most important mycotoxigenic fungi of maize kernels are *Fusarium* species, mainly *F. graminearum* and *F. verticillioides,* and *Aspergillus* section *Flavi*, mainly *A. flavus* (Chulze et al., 2014; Rodríguez, 2015).

Aspergillus flavus is a ubiquitous opportunistic fungal pathogen that infects developing maize kernels, peanuts, cotton and other economically important crops attacking plants that are weakened by environmental stresses such as drought and heat (Amare and Keller, 2014; Dolezal et al., 2014; Klich, 2007). This species includes isolates that produce different levels of aflatoxins. Aflatoxins are a group of about 20 chemically related metabolites produced primarily by *A. flavus* and *A. parasiticus*. Aflatoxins B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2) are the four major types. *A. flavus* isolates may produce AFB_1 and AFB_2, but neither AFG_1 nor AFG_2 (Atehnkeng et al., 2016; Perrone et al., 2014a). Aflatoxins contaminate a variety of staple foods including maize, peanuts, milk, dried fruits and tree nuts, and they are carcinogenic, teratogenic, immunosuppressive and genotoxic compounds that have been classified by the International Agency for Research on Cancer (IARC) as group 1 carcinogens (IARC, 2012). Aflatoxins may cause

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https://doi.org/10.1016/j.ijfoodmicro.2018.04.020
Received 16 November 2017; Received in revised form 26 March 2018; Accepted 13 April 2018
Available online 13 April 2018
0168-1605/

acute and chronic animal and human health disorders such as vomiting and abdominal pain typically resulting in death, acute toxic liver injury, hepatocellular carcinoma, adverse immune system effects and stunted growth in children (Atherstone et al., 2016; IARC, 2012; McMillan et al., 2018; Peraica et al., 1999; Williams et al., 2004; Wu, 2015). Besides aflatoxins, *A. flavus* may often produce cyclopiazonic acid (CPA), an indole tetramic acid that is toxic to a variety of animals and humans (Chang et al., 2009; Dorner et al., 1984). This mycotoxin has shown toxicity principally in chickens and pigs, and it has been implicated in human poisoning (Blaney et al., 1989; Rao and Husain, 1985). Toxic effects include liver degeneration and necrosis, myocardial lesions, decreased weight gain, vomiting, lesions of kidneys, pancreas, spleen and several neurotoxic symptoms (Duran et al., 2007; Kuilman-Wahls et al., 2002).

Mycotoxin production in *A. flavus* is highly variable and depends on several factors such as genotype, substrate and geographic origin, climate conditions, and agronomic practices. Moreover, *A. flavus* is more invasive in agricultural ecosystems and displaces *A. parasiticus* when both species are together in soil (Perrone et al., 2014a). Aflatoxins and CPA frequently co-occur in contaminated agricultural products such as maize and peanuts (Barros et al., 2005; Razzaghi-Abyaneh et al., 2006; Vaamonde et al., 2003), which may increase the toxicological risks since possible toxic synergies between these two mycotoxins could be important to animal health and potentially to human food safety (Maragos et al., 2017; Urano et al., 1992). Aflatoxins and CPA may also be transferred to other products such as milk and eggs (Abbas et al., 2006).

Aspergillus flavus populations include isolates with two morphologically distinct sclerotium size variants, L (large) strains with average sclerotium size > 400 μm in diameter and S (small) strains which produce numerous sclerotia < 400 μm in diameter in association with few conidial heads (Atehnkeng et al., 2008b; Cotty, 1989; Horn, 2007; Kachapulula et al., 2017; Perrone et al., 2014b). Both L and S strains have been isolated from different agro-ecosystems from Thailand, Australia, Benin, Italy, North America and Argentina (Blaney et al., 1989; Giorni et al., 2007; Pildain et al., 2004; Vaamonde et al., 2003) and they differ in some characteristics such as mycotoxin production (Cotty, 1994, 1997; Horn and Dorner, 1999; Mauro et al., 2015). It has been observed that *A. flavus* S strains are in general producers of higher levels of aflatoxins than L strains (Barros et al., 2005; Cotty, 1988, 1989; Garber and Cotty, 1997). Similarly, in recent studies Ehrlich (2014) determined that CPA is produced at higher levels by *A. flavus* S strains than L strains. Sclerotia are survival structures resistant to adverse environmental conditions. In addition, sclerotia of *A. flavus* germinate sporogenically on soil by producing aerial conidiophores, which represent a source of primary inoculum in crops (Horn et al., 2014). However, the role of sclerotia in the life cycle of *A. flavus* has been reconsidered since sexual stage associated with these structures was discovered (Horn et al., 2009).

Aspergillus flavus is heterothallic and strains typically contain one of two mating-type genes: *MAT 1-1* or *MAT 1-2*. Sexual reproduction in *A. flavus* happens between strains with opposite mating type (Moore et al., 2011; Olarte et al., 2012) and produces recombinant progeny through the independent chromosome distribution and through crossing over within the aflatoxin gene cluster as well as other portions of the genome (Horn et al., 2014). As consequence, recombination provides a high genetic variation in field populations of *A. flavus*, in which there may be found from non aflatoxigenic strains to high aflatoxin producers (Atehnkeng et al., 2008a; Barros et al., 2006; Bayman and Cotty, 1993; Dorner, 2004; Horn and Dorner, 1999; Horn, 2007).

Aflatoxins are highly regulated in human and animal food in more than 100 countries throughout the world (Wu, 2015). The MERCOSUR (Mercado Común del Sur) set a maximum limit for total aflatoxin content of 20 μg/kg in maize and derivates (MERCOSUR, 2002), and the European Union established a maximum level of 4 μg/kg for total aflatoxin in all cereals and derivates with the exception of maize to be subjected to physical treatment before human consumption, for which a maximum level for total aflatoxin of 10 μg/kg has been set (EC, 2010). The enforcement of these regulations causes loss of markets for agricultural products and reduced income at a global level (Wu, 2015).

Studies carried out in Argentina (Garrido et al., 2012) showed presence of aflatoxins in fresh-harvested and storage maize kernels during a 10-year survey. Other data on natural occurrence of mycotoxins have been published by Broggi et al. (2002), Chulze et al. (1989), González et al. (1999), Pacin et al. (2001, 2009), Resnik et al. (1996), Solovey et al. (1999) and others.

Climate change is predicted to have significant impacts on the quality and availability of staple food commodities (Medina et al., 2017). It is estimated that the global average temperature in the year 2100 will have increased in 4 °C, resulting in higher CO_2 and other atmospheric gas levels which would increase the frequency of dry conditions. All these factors would affect crop development and increase mycotoxin incidence (Ehrlich et al., 2015; Magan et al., 2011). It has been suggested that climate change may be responsible for up to a 1/3 of yield variability in key agricultural commodities on a global basis (Ray et al., 2015). This will have deep impacts on food security in different continents, including Argentina as one of the hot spots (Medina et al., 2017).

Several strategies to reduce aflatoxin contamination of agricultural commodities have included adjustments to planting date, irrigation, improved fertility management and fungicide application, but these have been only slightly effective (Weaver et al., 2016).

Biological control based on competitive exclusion achieved by applying competitive non aflatoxigenic strains of *A. flavus* and/or *A. parasiticus* to the soil of developing crops is based on the premise that spores of non aflatoxigenic strains compete with naturally occurring aflatoxigenic strains for infection sites and essential nutrients. This strategy for minimizing pre-harvest aflatoxin contamination of crops has been demonstrated under field conditions in cotton (Cotty, 1994), peanuts (Alaniz Zanon et al., 2013, 2016; Dorner et al., 2003; Dorner and Lamb, 2006; Pitt and Hocking, 2006) and maize (Abbas et al., 2006; Atehnkeng et al., 2008b, 2014). Knowledge of non aflatoxigenic *A. flavus* subpopulations in the maize growing region of Argentina may be useful in identifying local management practices to reduce aflatoxin contamination in maize. In this sense, the aims of the present study were to characterize a pool of *A. flavus* strains isolated from kernels and soil samples collected within the maize growing region in Argentina; and to evaluate the competitive ability of characterized non-aflatoxigenic *A. flavus* strains, CPA (+)/(−) as potential aflatoxin biocontrol agents.

2. Materials and methods

2.1. Aspergillus flavus strain isolation and identification

Thirty-one fields from different locations from Córdoba and San Luis provinces from the 2015/16 growing season were sampled. A total of 13 samples of maize kernels and 22 soil samples were collected. In 4 out of 31 fields both maize and soil samples were collected. *A. flavus* strains were isolated from both substrates.

Twenty ears were harvested from each one of the sampled fields and kernels were separated from cobs by hand. Maize kernels were superficially disinfected. Briefly, 100 kernels of each sample were immersed in 70% ethanol for 2 min, then in 0.4% chlorine during 2 min and rinsed with sterile distilled water for 1 min. Disinfected kernels were directly plated on Petri dishes containing Dichloran Rose Bengal Chloramphenicol agar (DRBC) (Pitt and Hocking, 2009).

From each of the 22 fields evaluated for soil, one soil sample was collected. Each soil sample was a pool of 5 sub-samples collected from the top 5 cm of soil along a diagonal line spanning each field. Samples were air-dried for 1–2 days at 25–30 °C, thoroughly mixed and passed through a sieve (2 mm mesh size). Ten grams of each sample were