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Cornmeal and starch influence the dynamic of fumonisin B, A and C production and masking in *Fusarium verticillioides* and *F. proliferatum*

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ABSTRACT

Fusarium verticillioides and *F. proliferatum* can infect maize ears and produce fumonisins. The fumonisin B (FB) series is the most prolifically produced, followed by fumonisin C (FC), A (FA) and P (FP); moreover hidden forms of fumonisins have been detected in maize and derivatives. There is a lack of information about which maize component may affect fumonisin pattern production. Therefore, in this work we studied the role of cornmeal and corn starch, as the sole source of nutrition, in the production dynamic of all fumonisin series, hidden forms included, in different strains of *F. verticillioides* and *F. proliferatum* incubated at 25 °C for 7–45 days. Both *Fusarium* species produced high amounts of FB, following the chemotype $FB_1 > FB_2 > FB_3$; FC and FA were produced in lesser amounts, showing the chemotypes: $FA_2 + FA_3 > FA_1$ and $FC_1 > FC_2 + FC_3 > FC_4$, respectively; while no FP were detected. *F. verticillioides* was more prolific than *F. proliferatum* in fumonisin production (ten times more on average) in all the tested conditions. Fumonisin production was higher in cornmeal than in starch based medium in both *Fusarium* species; FA and FC were detected only in the former medium. An important role of amylopectin as an inducing factor for fumonisin biosynthesis was suggested, as were acid pH conditions. Fumonisin hidden forms may occur in cornmeal medium, whereas they were never found at significant levels in corn starch medium.

1. Introduction

Maize is the second most cultivated crop worldwide (170 million ha) and has the highest average yield per hectare of any crop (~5.2 t/ha, in 2011). It is the most important agricultural commodity in the Americas and third most important, after wheat and barley, in Europe (faostat.fao.org). Several pests and diseases can reduce crop yield but one of the major concerns relates to *Fusarium verticillioides* and *Fusarium proliferatum*, the two fungi mainly responsible for pink ear rot on maize that can develop in almost all maize growing areas (Battilani and Logrieco, 2013). These fungi are known to produce fumonisins, mycotoxins that can accumulate in maize kernels during ripening. Fumonisins are considered a risk for human and animal health as fumonisin B_1 (FB_1) is known to cause equine leukoencephalomalacia, porcine pulmonary oedema, human oesophageal cancer and liver damage in swine (Howard et al., 2001; Marasas et al., 2004; Schroeder et al., 1996); it has been also classified as a possible carcinogen to humans (Group 2B) by the International Agency for Research on Cancer (IARC, 1993). Humans and animals are potentially exposed to fumonisin B (FB) worldwide and high levels of FBs are often reported in maize or maize-based food such as corn meal (Battilani et al., 2008; Bittencourt et al., 2005; Garrido et al., 2012; Silva et al., 2007).

Fumonisins B, and in particular FB_1 followed by FB_2, are the most abundant in nature (Rheeder et al., 2002). However these fungi are able to produce three minor series of fumonisin other than FB: fumonisin A (FA), fumonisin C (FC) and fumonisin P (FP). Fumonisin C and FA usually occur at lower concentrations than FB (<5%) (Bartók et al., 2006); FP can occur at levels up to 30% of FB when *F. verticillioides* is grown on cracked maize kernel medium (Musser et al., 1996). Fumonisins C are known to be phyto- and cytotoxic (Abbas et al., 1998) and they can co-occur with FB in naturally contaminated maize (Seo and Lee, 1999).

The in vitro production of fumonisin B is well studied (EHC, 2000; Marín et al., 1999; Scott, 2012). The dynamics of production of fumonisin series was studied by Lazzaro et al. (2012) in cultures of *F. verticillioides* grown on Malt Extract Agar for 45 days. They observed that FB, FA and FC, but no FP were produced. The production of all fumonisin series in *F. verticillioides* cultures was previously reported by Musser and Plattner (1997), but data on the production versus incubation time were not provided.

In addition to major and minor forms the occurrence of hidden fumonisins in maize has been reported (Dall'Asta et al., 2010; Dall'Asta et al., 2012). These forms are not covalently bound derivatives, they are entrapped in food macromolecules such as starch and proteins, thus they are determined with difficulty by common analytical methods. Hidden fumonisins cannot be fully regarded as masked mycotoxins, since the physical entrapment does not lead to a stable conjugation. In particular, masked mycotoxins are usually stable under gastrointestinal

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conditions (Dall'Erta et al., 2013), whereas parent fumonisins are totally released from hidden forms upon digestion (Dall'Asta et al., 2010; Falavigna et al., 2012b).

Nutritional factors are crucial for fumonisin production. Dried ripening maize kernels have the following composition (percentage on the maize seed weight): 65–80% starch, mainly concentrated in the endosperm, 1.4–2% soluble sugars, 8–10% protein and 3.5–4.5% oil, mainly located in the germ, 1.5–2.0% ash and 1.5–2.1% crude fibre, almost all in the pericarp, and 10–15% water (Dall'Asta et al., 2012; Smith et al., 2004).

Cornmeal is the main product in the diet of many populations living in South America and Africa, where it is considered a staple food and is often used to complement the diet of children (www.fao.org). Starch is also a significant product derived from maize and important in the human diet, especially in American countries. In the food industry, starch is used either as a thickening agent or as a precursor of sweeteners such as corn syrups (www.fao.org). Starch, whose major constituent is amylopectin (up to75% compared with 25% amylose, the other component), is the most abundant carbon source for fungi colonizing a ripening maize ear. Amylopectin is known to be an inducing factor for FB_1 production in *F. verticillioides* (Bluhm and Woloshuk, 2005).

As far as we are aware, there is a lack of information about which maize component could affect fumonisin pattern production and the masking phenomenon; only recently, a relation between fatty acids, linoleic acid in particular, and fumonisin production and masking was reported Dall'Asta et al. (2012). Contrary to masked mycotoxins such as deoxynivalenol-3-glucoside that can be formed exclusively by plant enzyme activity (Berthiller et al., 2012) hidden fumonisins may potentially be formed also under in vitro conditions, when unspecific supramolecular interactions may occur between fumonisins and medium macroconstituents.

For this reason we investigated the influence of corn meal and corn starch, as sole nutritional sources, on the production dynamic of all fumonisin series and on the masking phenomenon in *F. verticillioides* and *F. proliferatum* cultures.

This could provide more information regarding fungal metabolism, in terms of nutritional needs, and of the mechanism behind fumonisin production. Moreover it could benefit the understanding of the masking phenomenon, which has been observed only in maize and maize-based food, and whose mechanism has not yet been clarified.

2. Material and methods

2.1. Fungal material

The fungal species used in this study were *F. verticillioides* and the closely related species *F. proliferatum*. Three strains of *F. verticillioides* were considered: ITEM1744, ITEM10027 (MPVP 294) and ITEM10026 (MPVP 289), the first isolated from maize in South Sardinia, and the latter two in South Tuscany, Italy. Two strains of *Gibberella intermedia* (teleomorph of *F. proliferatum*) were included: ITEM 7595 and ITEM 15876 (MPVP328), respectively isolated from maize in Kansas, USA and the Po valley, Italy. Cultures are stored in 18% glycerol at the fungal collection of the Institute of Entomology and Plant Pathology-UCSC, Piacenza (MPVP) and at the Institute of Sciences of Food Production-CNR, Bari (ITEM; http://server.ispa.cnr.it/ITEM/Collection).

2.2. Inoculum preparation and maize-based substrates

All strains were grown on Potato Dextrose Agar at 25 °C for 7 d in the dark and at the end of incubation. A portion of the colony (7 mm Ø) was used as inoculum.

All strains were inoculated on two different solid media, cornmeal (CMM), made with cornmeal and sterile distilled water (1:7, volume) and corn starch (CSM), prepared with corn starch and sterile distilled water (1:1, volume). Commercial maize flour bought at the supermarket was used for CMM preparation whereas a commercial corn starch (Carlo Erba, Milano, Italy) was used for CSM.

Both substrates were tyndallised by heating for 30 min at 80 °C three times, poured into 90 mm Ø Petri plates and once cooled, covered by a cellophane sheet (P400; Cannings, Ltd., Bristol, UK) to facilitate removal of the fungal biomass. The fungal inoculum was added at the centre of the plate; a similar sized portion of uninoculated PDA was added to control plates. Cultures were incubated for 7, 14, 21, 30 or 45 days at 25 °C in the dark and then stored at −20 °C. The trial was performed in triplicate.

Water activity of both media was measured in triplicate using the Aqualab LITE (Decagon, Pullman, WA, USA), according to the manufacturer instructions, before inoculation of plates.

2.3. Sample preparation for free and total fumonisins

Samples were prepared according to Lazzaro et al. (2012). Briefly, aliquots (2 g) of the maize-based medium were blended in a high-speed blender (Ultraturrax T18; IKA, Stauffen, Germany) with 8 mL of water/methanol, 30:70 v/v, for 1 min and then centrifuged at 2470 *g* for 15 min. Next, an aliquot (50 μL) of supernatant was diluted with 450 μL of water/methanol, 30:70 v/v, before LC–ESI-MS/MS analysis.

Samples (2 g aliquot) for determination of hydrolysed fumonisins were blended with 20 mL of 2 M KOH, centrifuged for 1 min at 2474 *g* and then stirred for 60 min. The sample was then combined with 20 mL of acetonitrile, stirred for 10 min, and then centrifuged at 2470 *g* for 15 min in order to resolve the sample into two layers. A 200 μL portion of the upper, acetonitrile-rich layer was evaporated to dryness under a stream of compressed air, and the residue was re-dissolved in 800 μL water/methanol, 30:70 v/v and analysed by LC–ESI-MS/MS. After hydrolysis, samples were examined by LC–ESI-MS/MS for the presence of the hydrolysed forms of FB_1, FB_2 and FB_3.

All the results are expressed as the sum of FB_1, FB_2, and FB_3 equivalents, considering a correction factor due to the different molecular weights of parent and hydrolysed compounds and referred to as "total fumonisins after hydrolysis".

2.4. LC–ESI-MS/MS analysis for the determination of free and total fumonisins

Free and total fumonisins were determined according to Dall'Asta et al. (2010) without any modification.

Very briefly, LC–ESI-MS/MS analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a Quattro API triple quadrupole mass spectrometer with an electrospray source (Micromass; Waters, Manchester, UK). Chromatographic conditions were set as follows: the column was a 250 mm × 2.1 mm i.d., 5 μm, XTerra C18; the flow rate was 0.2 mL/min; the column temperature was set at 30 °C; the injection volume was 10 μL; gradient elution was performed using bidistilled water (eluent A) and methanol (eluent B) both acidified with 0.2% formic acid: initial condition at 70% A, 0–2 min isocratic step, 2–5 min linear gradient to 45% B, 5–25 min linear gradient to 90% B, 25–35 min isocratic step at 90% B, 35–36 min linear gradient to 70% A, and reequilibration step at 70% A for 15 min (total analysis time: 50 min). MS parameters: ESI + (positive ionisation mode); capillary voltage, 4.0 kV; cone voltage, 50 V; extractor voltage, 2 V; source block temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow and desolvation gas flow (nitrogen), 50 L/h and 700 L/h, respectively.

Detection was performed using a multiple reaction monitoring (MRM) mode by monitoring two transitions for each analyte, as follows: 722.4 → 334.4, 722.4 → 352.3 for FB_1, 706.4 → 336.4 and 706.4 → 318.4 for FB_2 and FB_3, 406.5 → 370.5 and 406.5 → 388.5 for HFB_1, 390.5 → 336.4 and 390.5 → 372.5 for HFB_2 and HFB_3, 564.1 → 334.4 and 564.1 → 352.2 for $PHFB_1$, 548.2 → 336.4 and 548.2 → 354.20 for $PHFB_2$ and $PHFB_3$, 532.2 → 338.2 and 532.2 → 320.2 for $PHFB_4$.