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Environmental factors related to fungal infection and fumonisin accumulation during the development and drying of white maize kernels

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ABSTRACT

In Southern Europe where whole maize kernels are ground and used for making bread and other food products, infection of the kernels by *Fusarium verticillioides* and subsequent fumonisin contamination pose a serious safety issue. The influence of environmental factors on this fungal infection and mycotoxin accumulation as the kernel develops has not been fully determined, especially in such food grade maize. The objectives of the present study were to determine which environmental factors may contribute to kernel invasion by *F. verticillioides* and fumonisin accumulation as kernels develop and dry in naturally infected white maize. Three maize hybrids were planted at two different sowing dates and kernel samples were collected 20, 40, 60, 80 and 100 days after silking. The percentage of kernels infected, and ergosterol and fumonisin contents were recorded for each sampling. *F. verticillioides* was the most prevalent species identified as the kernels developed. Temperature and moisture conditions during the first 80 days after silking favored natural kernel infection by *F. verticillioides* rather than by *Aspergillus* or *Penicillium* species. Fumonisin was found in kernels as early as 20 days after silking however significant fumonisin accumulation above levels acceptable in the EU did not occur until after physiological maturity of the kernel indicating that kernel drying in the field poses a high risk. Our results suggest that this could be due to increasing kernel damage by insects that favor fungal development, such as the damage by the moth *Sitotroga cerealella*, and to the occurrence of stress conditions for *F. verticillioides* growth that could trigger fumonisin biosynthesis, such as exposure to suboptimal temperatures for growth simultaneously with low water activity.

1. Introduction

Fusarium verticillioides (Sacc.) Nirenberg can infect maize (*Zea mays* L.) at most stages of the plant's development and growth (Bacon et al., 2008). Before silking, *F. verticillioides* infection is mostly localized in basal organs such as the stalk; but at silking, silks become the most important pathway for *F. verticillioides* to enter the ear and a general increase in infection can be observed throughout the plant especially in tissues such as glumes and husks (Munkvold et al., 1997; Venturini et al., 2011). After glume colonization, *F. verticillioides* can use the open stylar canal to enter into unwounded kernels (Duncan and Howard, 2010). Asymptomatic infection is common throughout the maize plant. Disease development can result in poor stand establishment, stalk rot, and kernel infection with the latter posing a serious economic threat as this fungus can contaminate the kernels with fumonisin mycotoxins (Munkvold and Desjardins, 1997).

Fumonisins are among the most prevalent mycotoxins in maize and maize-based food and feed in Southern Europe (Binder et al., 2007; EHC, 2000). Many fumonisin analogs have been characterized, but fumonisin B_1 (FB_1) typically accounts for 70 to 80% of the total fumonisins found, and fumonisin B_2 (FB_2) makes up the 15 to 25% (Rheeder et al., 2002). Fumonisin toxicity is related to their capacity to disrupt the biosynthesis of sphingolipids, the main components of the plasmatic membrane of cells, resulting in apoptosis and disturbances of cellular processes such as cell growth, cell differentiation and morphology, and endothelial cell permeability (SCF, 2000; Voss et al., 2007). In humans, fumonisins are suspected risk factors for esophageal cancer and neural tube defects (Bennett and Klich, 2003) and the International Agency for Research on Cancer has classified them as probably carcinogenic (IARC, 1993). In livestock, fumonisins cause leukoencephalomalacia in horses, pulmonary edema in pigs, reduced growth in poultry and hepatic and immune disorders in cattle (Logrieco et al., 2003; Voss et al., 2007).

In a recent review, Picot et al. (2010) reported that eco-physiological factors such as water activity and temperature, physiochemical and nutritional factors such as pH and C:N ratio, and carbon metabolism, and plant defense metabolites such as oxylipins and phenolic compounds are important factors for regulating fumonisin production under laboratory conditions. Warfield and Gilchrist (1999) studied the dynamics of *F. verticillioides* infection and fumonisin accumulation during kernel

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development using inoculation on detached kernels. They showed that fumonisin production significantly increased with kernel development with levels of FB_1 being the highest at the dent stage and lowest at the earlier blister stage. It was concluded that toxin production was affected by substrate composition as well as by moisture content suggesting that changes in kernel composition during kernel maturation "may represent a developmental transition in signaling metabolites within the developing kernel which could also play a role in regulating FB_1 synthesis". However, kernels detached before reaching physiological maturity are nonviable. The crosstalk between the host and pathogen is disrupted in such kernels thus interfering in the outcome of a plant–pathogen interaction, as stated by Mukherjee et al. (2011) who observed different FB_1 production responses on nonviable versus viable kernels.

F. verticillioides infection and fumonisin accumulation as kernels develop in field grown maize have been described, but little information about biotic and abiotic factors influencing infection and toxin accumulation has been published (Almeida et al., 2002; Bush et al., 2004; Chulze et al., 1996; King, 1981; Zorzete et al., 2008). Picot et al. (2011) suggested that fumonisin production can be initiated during the dough stage, which correspond approximately to 60 to 70% kernel moisture, but physiological changes occurring during the dent stage, such as amylopectin and pH modifications, may enhance fumonisin biosynthesis. The influence of environmental factors other than those related to kernel composition or physiological characteristics, on kernel infection by *F. verticillioides* and fumonisin accumulation in field corn during kernel development and drying has not been thoroughly studied. The objectives of the present work were: 1) to monitor kernel invasion by *F. verticillioides* and the subsequent contamination with fumonisin under field conditions of natural inoculation; and 2) to search for environmental factors related to fumonisin accumulation during kernel development and drying in white maize. A few studies have evaluated yellow maize and white maize at the same time in relation to fumonisin contamination, and most of them showed inconclusive results relating to differences in contamination (Fadohan et al., 2003; Clements et al., 2004; Kleinschmidt et al., 2005). Our focus in the current research was on white maize, which is traditionally ground and used for making bread and other bakery products in the northwest region of the Iberian peninsula of Spain (Butrón et al., 2009). Fumonisin contamination of this maize could pose a considerable health threat and must be mitigated.

2. Materials and methods

2.1. Field evaluations

Three white maize hybrids (EP10xEC22, EP65xEP10 and EP71xEC22) were chosen for evaluation based on their different levels of fumonisin contamination in a previous study (Butron et al., 2006). In 2009, the hybrids were hand-planted at two different sowing dates (early and late May) in Pontevedra (42°24′ N, 8°38′ W, 20 m above sea level), Northwestern Spain. The late planting date was 23 days after the early planting. Hybrids silked in mid-July and early August, for the early and late plantings, respectively. The experimental design for each planting date was a split-plot with three replications. Hybrids were assigned to the main plots and sampling dates (20, 40, 60, 80 and 100 days after silking) to the subplot units. Each plot consisted of one row with 29 plants spaced 0.21 m apart. The distance between adjacent rows was 0.8 m. Rows were overplanted and thinned to obtain a final plant density of about 60,000 plants/ha.

Within each plot, five ears (subplot) were randomly collected at each sampling date and data was recorded on: husk tightness using a visual rating scale from 1 (loose husks with visible cob) to 5 (tight husks) [1 = 0% tight, 2 = 30%, 3 = 50%, 4 = 70% and 5 = 100% tight husks] (Wiseman and Isenhour, 1992); damage from boring insects [*Sesamia nonagrioides* (Lefèbvre) and *Ostrinia nubilalis* (Hübner)] using a visual rating scale from 1 (ear totally damaged by borers) to 9 (no damage) [1 = > 90% damaged, 2 = 81–90% damaged, 3 = 71–80% damaged, 4 = 61–70% damaged, 5 = 41–60% damaged, 6 = 31–40% damaged, 7 = 21–30% damaged, 8 = 1–20% damaged and 9 = 0%] (Sandoya et al., 2010); Fusarium ear rot using a similar visual rating scale from 1 (total ear visually infected) to 9 (no symptoms of infection); damage by *Sitotroga cerealella* (Oliver) measured as number of kernels perforated by the larvae; and, kernel moisture (by calculating the difference between the fresh and the dry weight of a grain subsample of approximately 100 g, after drying until constant weight at 80 °C for 4–6 days). Grain was dried at 35°°C for one week and maintained at 4 °C until biological and chemical analyses could be conducted.

Several climatic variables were calculated for the 20-day period preceding each sampling date including: average daily mean temperature (°C), average daily maximum temperature (°C), average daily minimum temperature (°C), average daily mean relative humidity (%), daily mean precipitation (mm), number of days with minimum temperature ≤15 °C, number of days with maximum temperatures ≥30 °C, number of days with mean temperature ≥10 °C and <15 °C, ≥15 and <20 °C, ≥20 and <25 °C, and ≥25 and <30 °C, and number of days with rainfall ≥2 mm. These climatic variables were selected according to previous reports on the influence of climatic factors on mold development in wheat and maize (de la Campa et al., 2005; Maiorano et al., 2009; Marin et al., 2004; Schaafsma and Hooker, 2007).

2.2. Determination of fungal species infecting maize kernels

On each of the five-ear samples, the percentage of kernels infected by molds was computed and the fungal genera and *Fusarium* species were determined. Analyses of fungal infection and ergosterol were not carried out with samples from the first sampling date of the early planting because those samples were dried at 60 °C and that temperature disturbed kernels' and fungal tissues' integrity. From each other sample, one hundred kernels were externally disinfected with 3% sodium hypochlorite. Fifty disinfected kernels were incubated at 25 °C for five days on Petri dishes containing DRBC (Dichloran rose-bengal chloramphenicol agar) culture medium in order to determine the percentage of kernels infected by molds (King et al., 1979; Van Pamel et al., 2009). The isolates of *Penicillium* and *Aspergillus* were identified and counted (Pitt and Hocking, 2009). The remaining disinfected 50 kernels were incubated at 25 °C for six days on Petri dishes containing MGA (Malachite Green Agar) culture medium for isolation of *Fusarium* species (Alborch et al., 2010; Castella et al., 1997). The isolates were counted and grouped according to cultural and microscopic features of the mycelium, thereafter transferred to Petri dishes containing SNA (Spezieller Nährstoffarmer agar) culture medium (Leslie and Summerell, 2006) and incubated at 25 °C for seven days (12:12 h of day:night light conditions). A small amount of mycelium from each Petri dish was added to 10 ml of distilled water and vortexed; the resulting spore suspension was poured and spread on a Petri dish containing water-agar (20 g/l of agar) culture medium. Petri dishes were inclined and incubated at 25 °C for 16–18 h for favoring the formation of a spore gradient. Then, a single spore was isolated from each dish, transferred to a Petri dish containing SNA medium and incubated at 25 °C for 15 days (12:12 h of day:night light conditions) to allow mycelia growth and subsequent identification of the *Fusarium* species. A small scrape of mycelium was spread in a tube that contained PDA medium and incubated for 7 days at 25 °C (12:12 h of day:night light conditions). Identification was performed taking into account microscopic morphological characteristics of the mycelium and spores on SNA culture medium and coloration on PDA medium (Leslie and Summerell, 2006).

2.3. Ergosterol and fumonisin quantifications

Ergosterol and fumonisin analyses from each subplot were performed on representative 10 g sample taken from 200 g of dried ground kernels which had been ground through a 0.75 mm screen