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4 **Enviromental Factors Related to Fungal Infection and Fumonisin Accumulation during the**
5 **Development and Drying of White Maize Kernels**

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15 **Abstract**

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

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45 **Keywords:** Maize; *Zea mays* L.; *Fusarium verticillioides*; fumonisin; kernel development.

46 1. Introduction

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

56 Fumonisins are among the most prevalent mycotoxins in maize and maize-based food and feed in Southern
57 Europe (Binder et al., 2007; EHC, 2000). Many fumonisin analogs have been characterized, but fumonisin B₁
58 (FB₁) typically accounts for 70 to 80% of the total fumonisins found, and fumonisin B₂ (FB₂) makes up from 15
59 to 25% (Rheeder et al., 2002). Fumonisin toxicity is related to their capacity to disrupt the biosynthesis of
60 sphingolipids, the main components of the plasmatic membrane of cells, resulting in apoptosis and disturbances
61 of cellular processes such as cell growth, cell differentiation and morphology, and endothelial cell permeability
62 (SCF, 2000; Voss et al., 2007). In humans, fumonisins are suspected risk factors for esophageal cancer and neural
63 tube defects (Bennet and Klich, 2003) and the International Agency for Research on Cancer has classified them
64 as probably carcinogenic (IARC, 1993). In livestock, fumonisins cause leukoencephalomalacia in horses,
65 pulmonary edema in pigs, reduced growth in poultry and hepatic and immune disorders in cattle (Logrieco et al.,
66 2003; Voss et al., 2007).

67 In a recent review, Picot et al. (2010) reported that eco-physiological factors such as water activity and
68 temperature, physiochemical and nutritional factors such as pH and C: N ratio, and carbon metabolism, and
69 plant defense metabolites such as oxylipins and phenolic compounds are important factors for regulating
70 fumonisin production under laboratory conditions. Warfield and Gilchrist (1999) studied the dynamics of *F.*
71 *verticillioides* infection and fumonisin accumulation during kernel development using inoculation on detached
72 kernels. They showed that fumonisin production significantly increased with kernel development with levels of
73 FB₁ being the highest at the dent stage and lowest at the earlier blister stage. It was concluded that toxin
74 production was affected by substrate composition as well as by moisture content suggesting that changes in
75 kernel composition during kernel maturation “may represent a developmental transition in signaling metabolites
76 within the developing kernel which could also play a role in regulating FB₁ synthesis”. However, kernels
77 detached before reaching physiological maturity are nonviable. The crosstalk between the host and pathogen is
78 disrupted in such kernels thus interfering in the outcome of a plant–pathogen interaction, as stated by Mukherjee
79 et al. (2011) who observed different FB₁ production responses on nonviable versus viable kernels.

Fusarium verticillioides infection and fumonisin accumulation as kernels develop in field grown maize has 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. Scarcely studies evaluated yellow and white maize at the same time in relation to fumonisin contamination, and most of them showed inconclusive results attending to differences in contamination (Fadohan et al., 2003; Clements et al., 2004; Kleinschmidt et al., 2005). Our focus in the current research was on human food 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 (Butrón 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 *Ostinia 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

115 and 9 = 0%](Sandoya et al., 2010); Fusarium ear rot using a similar visual rating scale from 1 (total ear visually
116 infected) to 9 (no symptoms of infection); damage by *Sitotroga cerealella* (Oliver) measured as number of kernels
117 perforated by the larvae; and, kernel moisture (by calculating the difference between the fresh and dry weight of
118 a grain subsample of approximately 100 g, after drying until constant weight at 80 °C for 4-6 days). Grain was
119 dried at 35° C for one week and maintained at 4°C until biological and chemical analyses could be conducted.

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

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129 2.2. Determination of fungal species infecting maize kernels

130 On each of the five-ear samples, the percentage of kernels infected by molds was computed and the fungal
131 genera and *Fusarium* species were determined. Analyses of fungal infection and ergosterol were not carried out
132 with samples from the first sampling date of the early planting because those samples were dried at 60 °C and
133 that temperature disturbed kernels and fungal tissues integrity. From each other sample, one hundred kernels
134 were externally disinfected with 3% sodium hypochlorite. Fifty disinfected kernels were incubated at 25 °C for
135 five days on Petri dishes containing DRBC (Dichloran rose-bengal chloramphenicol agar) culture medium in
136 order to determine the percentage of kernels infected by molds (King et al, 1979; Van Pamel et al., 2009). The
137 isolates of *Penicillium* and *Aspergillus* were identified and counted (Pitt et al. 2009). The remaining disinfected 50
138 kernels were incubated at 25 °C for six days on Petri dishes containing MGA (Malachite Green Agar) culture
139 medium for isolation of *Fusarium* species (Alborch et al., 2010; Castellá et al., 1997). The isolates were counted
140 and grouped according to cultural and microscopic features of the mycelium, thereafter transferred to Petri
141 dishes containing SNA (Spezieller Nährstoffarmer agar) culture medium (Leslie and Summerell, 2006) and
142 incubated at 25 °C for seven days (12:12 hours of day: night light conditions). A small amount of mycelium from
143 each Petri dish was added to 10 ml of distilled water and vortexed, the resulting spore suspension was poured
144 and spread on a Petri dish containing water-agar (20 g /l of agar) culture medium. Petri dishes were inclined and
145 incubated at 25 °C for 16-18 hours for favoring the formation of a spore gradient. Then, a single spore was
146 isolated from each dish, transferred to a Petri dish containing SNA medium and incubated at 25 °C for 15 days
147 (12:12 hours of day: night light conditions) to allow mycelia growth and subsequent identification of the *Fusarium*
148 species. A small scrape of mycelium was spread in a tube contained PDA medium and incubated for 7 days at 25
149 °C (12:12 hours of day: night light conditions). Identification was performed taking into account microscopic

150 morphological characteristics of the mycelium and spores on SNA culture medium and coloration on PDA
151 medium (Leslie and Summerell, 2006).

152 2.3. Ergosterol and fumonisin quantifications

153 Ergosterol and fumonisin analyses from each subplot were performed on representative 10 g sample taken from
154 200 g of dried ground kernels which had been ground through a 0.75 mm screen in a Pulverisette 14 rotor mill
155 (Fritsch GmbH, Oberstein, Germany). For ergosterol analysis, 50 ml of methanol (HPLC grade) were added to
156 10 g of maize flour; the mixture was shaken for 30 minutes and then filtered through a sieve of filter paper. Ten
157 ml of the filtered solution was mixed with 1.2 g of potassium hydroxide. Ergosterol extraction was performed
158 twice with 10 ml of hexane in a water bath at 55-60 °C for 30 minutes. The upper layers were recovered,
159 combined and evaporated in a rotary evaporator at 40°C. Extracts were dissolved in methanol (HPLC grade),
160 transferred to a vial and evaporated under a gentle N₂ flow in a sample concentrator (Stuart, Bibby Scientific
161 Limited, Staffordshire, UK). Samples were dissolved in 1 ml of methanol (HPLC grade) prior to HPLC analysis.

162 HPLC separation was carried out in a Waters HPLC-system (Waters 2695, separations module, Waters
163 Corporation, Milford, USA) at room temperature by injecting a 100 µL sample onto a C18 column (Waters
164 Spherisorb ODS2, 250 x 4.6mm, 5µm) at a flow rate of 1 ml/min with methanol (HPLC grade) in isocratic
165 conditions. Detection of ergosterol was made using an absorbance detector (Waters 2487 dual λ absorbance
166 detector, Waters Corporation, Milford, USA) set at 282 nm. Quantification was performed using external
167 calibration with ergosterol standard solutions (Sigma, St. Louis, MO, USA) ranging from 0.08 to 5 µg/ml.
168 Detection limit of the analysis was 0.013 µg/g.

169 Fumonisin extraction from the 10 g samples was made with a solvent of 50 ml of distilled water: methanol:
170 acetonitrile (50:25:25) and 1 g of sodium chloride. The mixture was agitated for 20 minutes and filtered through a
171 sieve of filter paper. Ten milliliters of the filtered solution were suspended on 40 ml of PBS. The resulting 50 ml
172 were passed through an immunoaffinity column (Fumoniprep, R-Biopharm Rhône Ltd, UK) and fumonisins
173 were recovered using 1.5 ml of methanol and 1.5 ml of MiliQ water. Fumonisin quantification was performed in
174 a Waters HPLC-system (Waters 2695, separations module, Waters Corporation, Milford, USA) equipped with
175 fluorescence detector (Waters Multi λ Fluorescence Detector 2475, excitation λ at 335 nm and emission λ at 440
176 nm) and a C18 column (Waters Spherisorb ODS2, 150 mm x 4.6 mm, 5 µm) connected to a precolumn. One
177 hundred µl were injected into the HPLC system after derivatization of fumonisins with *o*-phthaldialdehyde, at 30
178 °C and a flow rate of 1 ml/min. The mobile phase was methanol: 0.1 M sodium dihydrogen phosphate (77:23).
179 Quantification was performed using external calibration with FB₁ and FB₂ standard solutions (Sigma, St. Louis,
180 MO, USA), ranging from 0.08 to 2.5 µg/ml. Results were converted into µg/g of dry maize flour. Detection
181 limits for FB₁ and FB₂ were 0.02µg/g and 0.08 µg/g, respectively.

182 Ergosterol, FB₁ and FB₂ concentrations in a fresh sample were calculated by multiplying the concentrations in
183 the dry kernel sample by the percentage of dry weight on the total weight (fresh sample weight).

184 2.4. Statistical analyses

185 Individual and combined analyses of variance were performed using the PROC GLM procedure of SAS (SAS,
186 2008) for most of the traits evaluated. Hybrid, sampling and planting dates were considered as fixed factors.
187 Fumonisin concentrations were log-transformed as $\log(x+1)$ to ensure normal distribution of residues and
188 homogeneity of variance. Mean comparisons were made using the Fisher's least significant difference (LSD) at
189 0.05 probability level. Husk tightness, borer damage and Fusarium ear rot ratings were rank transformed and
190 analyzed by a nonparametric analysis of ordinal data (Shah and Madden, 2004).

191 Pearson's correlation coefficients were computed with data averaged across replications and hybrids using the
192 PROC CORR procedure of SAS (n=10). To explain changes in the rate of kernel infection by *F. verticillioides* and
193 fumonisin accumulation with kernel development, we used multiple linear regression on differentials between
194 consecutive harvests for kernel characteristics, and climatic variables calculated for the 20-days period before
195 each kernel sampling date (20, 40, 60, 80, and 100 days after silking) in two planting dates (n=8). For regression
196 analysis, we used increments between consecutive harvests rather than data at each sampling date because
197 infection and fumonisin content increased with time and that could generate spurious correlations between these
198 data and environmental characteristics that also change with time. We used stepwise selection with the PROC
199 REG procedure of SAS for performing multiple linear regressions.

200 3. Results

201 There were significant differences between planting dates for the percentage of kernels infected by *Aspergillus*,
202 husk tightness, and kernel damage by *S. cerealella* (data not shown). There were no significant differences among
203 hybrids for fumonisin concentrations, but there were for husk tightness, kernel damage by *S. cerealella*, Fusarium
204 ear rot and kernel moisture (data not shown). There were significant differences among kernel samples collected
205 at the different sampling dates (20, 40, 60, 80 and 100 days after silking) for all traits, except the percentage of
206 kernels infected by *Penicillium* and *Fusarium proliferatum* (Matsushima) Nirenberg (Table S1).

207 *Fusarium* was the most prevalent genus at each sampling date, with *F. verticillioides* being the species most isolated
208 (Figure 1, Table S1). The percentage of kernels infected by *F. verticillioides*, *Fusarium* spp. other than *F. verticillioides*
209 or *F. proliferatum*, and by *Aspergillus*, significantly changed with kernel development. The number of kernels
210 infected by *F. verticillioides* significantly decreased from 20 to 40 days after silking, but when kernel development
211 was completed, the percentage of kernels infected increased linearly until reaching 70% at 100 days after silking.
212 Another *Fusarium* infection peak occurred at 80 days after silking (20.8%). Kernel infection caused by *Aspergillus*
213 and *F. proliferatum* peaked at 60 days after silking (2%) then decreased as the kernels dried. *Penicillium* infection
214 occurred at every kernel stage with percentages varying between 1 and 8%. Ergosterol content in the kernel
215 showed a non significant tendency to decrease from 20 to 40 days after silking, a linear and significant increase
216 from 40 to 80, and a sharp increase from 80 to 100 days after silking (Figure 1, Table S1). Fumonisin were
217 detected in kernels at 20 days after silking and significantly increased with sampling dates (Table 1). Husk

218 tightness significantly decreased with kernel development. Kernel damage by *S. cerealella* and Fusarium ear rot
219 significantly increased with kernel development.

220 The interaction between planting date $\times$ sampling (or kernel development stage) was significant for FB₁, FB₂,
221 total FB, borer and Angoumois grain moth damage, and percentage of kernel infected by *Aspergillus* (Table 1).
222 Total fumonisin content were similar until 60 days after silking for both planting dates; however, significant
223 increase in fumonisins occurred 60 to 80 days after silking in the late planting trial and 80 to 100 days after
224 silking in the earlier planted trial. Nevertheless, fumonisin contents in kernels 100 days after silking were
225 significantly higher in the early than in the late planted trial. For this study, fumonisin data were based on kernel
226 fresh weight to minimize kernel weight changes with time, but food and feed safety levels are always based on
227 dry matter content. Thus, our fumonisin concentrations when based on dry matter content were quite high with
228 levels of 13.99 $\mu\text{g/g}$ and 7.16 $\mu\text{g/g}$ at 100 days after planting for the early and late trial, respectively. Kernel
229 damage by *S. cerealella* increased with kernel development and drying in both planting trials but was significantly
230 higher for all sampling dates in the earlier planted trial. Damage to the ears by corn borers was higher in the late
231 planting at increasing kernels age (Table 1).

232 Since sampling time and planting date had significant effect on many of the traits, correlation and regression
233 analyses were performed with mean data for each sampling $\times$ planting date combination. The simple correlation
234 coefficient between percentage of kernels infected by molds and percentage of kernels infected by *F. verticillioides*
235 was extremely high (Table 2). The percentage of kernels infected by the genus *Fusarium* and the species *F.*
236 *verticillioides* were significantly correlated with ergosterol content, although Fusarium ear rot was significantly
237 correlated with kernel infection by *Fusarium*, *F. verticillioides* and other *Fusarium*. Fumonisin content was correlated
238 with the percentage of kernels infected by *F. verticillioides*, husk tightness and kernel moisture. Ergosterol and
239 fumonisin contents were highly correlated ($r=0.93$) and both showed significant association with kernel damage
240 by *S. cerealella* and Fusarium ear rot.

241 The climatic variables calculated for the 20-day period before kernel sampling dates (20, 40, 60, 80 and 100 days
242 after silking) in two planting dates are shown in table 3. In order to minimize the time effect on variables, we
243 used the differentials of biotic and abiotic variables between consecutive sampling dates and used regression
244 analysis to explore the effect of these variables on *F. verticillioides* infection, and fumonisin and ergosterol contents
245 (Table 3). Variability for differentials of the percentage of kernels infected by *F. verticillioides* between consecutive
246 harvests were associated with variability of changes between consecutive harvests for days with maximum
247 temperature $\geq 30^\circ\text{C}$, and days with rainfall ≥ 2 mm. Increasing damage by *S. cerealella*, explained 49% of
248 variability for the rate of fungal growth (differential for ergosterol content between consecutive harvests). An
249 increase in ergosterol rate and higher decreases for days with mean temperature between 15 and 20 $^\circ\text{C}$ explained
250 the 87% and 6%, respectively, of the variability for the rate of fumonisin accumulation. In addition, when
251 ergosterol content was not included in the stepwise model, increase in the differential for days with mean

temperatures between 10 and 15 °C, and the decrease of the differential for daily mean rainfall explained the 89% of variability for the rate of fumonisin increase (Table 4).

4. Discussion

4.1. Fungal infection

The genus *Fusarium* was a prevalent fungus at all kernel development stages sampled in this study with kernel infection by *Fusarium* representing more than 80% of the fungal infections. Infection by *Penicillium*, *Aspergillus* and other fungal species were marginal at each kernel development stage. *F. verticillioides* was the most abundant species, in agreement with previous reports in Northwestern Spain and Southern Europe (Butrón et al., 2006; Logrieco et al., 2002). Mean daily temperatures between 15 and 20 °C and high kernel moisture until 80 days after silking [corresponding to water activities ranging from 1 to 0.95, according to Maiorano et al. (2010)] are considered to be more favorable conditions for natural kernel infection by *F. verticillioides* than for *Aspergillus* and *Penicillium* species (Marín et al., 2004).

With respect to the progress of infection as the kernels developed, our results associated increases in the incidence of *F. verticillioides* with decreases in the number of days with extreme high temperatures (≥ 30 °C), and also with increases in the number of days with appreciable rainfall (≥ 2 mm). These weather conditions could favor spore production and dispersal. Rossi et al. (2009) reported that sporulation by *F. verticillioides* progressively increased between 5 °C and 27 °C and then declined rapidly with temperatures higher than 30 °C being less favorable for spore production. The number of rainy days also had a positive relationship to spore production, and rainfall and splashing favored spore dispersal (Ooka and Kommedhal, 1977; Rossi et al., 2009).

4.2. Fungal growth

As most of fungal isolates belonged to the species *F. verticillioides* and the percentage of kernels infected by *F. verticillioides* was significantly correlated with ergosterol content (unlike non-*Fusarium* infections) we used the amount of this sterol found in fungal membranes as an indirect measurement of *F. verticillioides* development in this study. At milk stage (20 days after silking, approximately 80% kernel moisture), maize ears were already infected by *F. verticillioides*, in accordance with previous studies; however, in our study, the percentage of infected kernels at this time was high in comparison to that described previously (Bush et al., 2004; King, 1981). In the subsequent 20-day period, a decrease in the number of infected kernels and low ergosterol content was observed in contrast with observations reported previously. Picot et al. (2011) reported an important change in fungal growth during the transition from the milk to the dent kernel stage (40 days after silking, approximately 50% kernel moisture) and suggested that, in general, *F. verticillioides* did not further colonize the maize ears after 42 days from inoculation (46-50 days after silking, approximately). Nevertheless, in our experiment, *F. verticillioides* growth, measured as the rate of ergosterol content increase, was almost linear from 40 to 80 days after silking after which the increase was even higher between 80 and 100 days. Our results showed that environmental characteristics, besides those directly related to kernel changes, could play an important role in fungal

development. With kernel development, increased differentials for ergosterol content between consecutive harvests were favored by increased rate of kernel damage by the larvae *S. cerealella*. The activity of insects can facilitate the dispersion and entry of fungus into maize kernels and damaged kernels may have increases in kernel moisture contents thus providing even better conditions for fungal development (Imura and Sinha, 1984; Misra et al., 1961).

4.3. Fumonisin production

According to Nielsen (2001), kernels harvested 20, 40, and 60 days after silking corresponded to the blister-milk, dent, and physiological maturity stages, since our kernel moistures were 80-83 %, 48-49 %, and 31-32 %, respectively. In previous studies, the dent stage has been reported as the stage most conducive to fumonisin production (Picot et al., 2011; Warfield and Gilchrist, 1999), and fluctuating fumonisin contents have been reported with kernel drying (Bush et al., 2004; Picot et al., 2011). However, our results showed that the pattern of fumonisin accumulation did not change or changed very little from the blister-milk stage to physiological maturity, and increased during the kernel drying period, especially at the end of our sampling period. In the laboratory study by Warfield and Gilchrist (1999), it is possible that different treatments (autoclaved / not autoclaved) applied to kernels from different stages and the use of detached ears could alter the physical and chemical kernel properties or the natural crosstalk between the plant and the fungus, with consequences on fumonisin production (Marín et al., 2004; Mukherjee et al., 2011). In addition, differences between field studies could be attributable to differences in sampling frequencies, fumonisin measurements (ELISA or HPLC), maize genotypes, and/or climatic factors (Shephard et al., 1996; Bush et al., 2004; Kleinschmidt, 2005; Battilani et al., 2011). Mean temperatures gradually decreased with kernel development in our trials in Northwestern Spain; while, in Southwestern France, a rapid decrease of the mean temperature below 15 °C was reached around physiological maturity (Picot et al., 2011) making drying conditions unfavorable for fumonisin production. On the other hand, fumonisin accumulation should be estimated per kernel as kernel weight changes with time. However, when fumonisin accumulation is based on fresh weight, as in this study, the bias is lower than when it is based on dry weight, as in the study by Picot et al. (2011), since fresh weight changes are significantly lower than dry weight changes with time (Bulant et al., 2000).

The simple correlation coefficient between kernel fumonisin and ergosterol contents and between fumonisin and the percentage of kernels infected by *F. verticillioides* were highly significant ($r=0.97$ and 0.67 , respectively). A steady accumulation of fumonisins from 20 to 60 days after silking accompanied a drop in the percentage of kernels infected by *F. verticillioides* and in ergosterol content observed at 40 days after silking, while a more rapid accumulation of fumonisin beyond 60 days after silking preceded the ergosterol burst in the period of 80-100 days after silking. Mycotoxins could be competitive factors against other fungal species, however fumonisin production itself has not been directly associated with such a competition strategy (Marín et al. 2001, 2004). The results of our study point to fumonisins being involved in response to plant stress. The stimulation of mycotoxin formation under growth stress conditions as a result of temperature and water extremes has long been postulated

321 (Samapundo et al., 2005). Kim et al. (2011) reported that enzymes implicated in sugar sensing/signaling networks
322 for controlling growth and development in response to the changing environment also have an important role
323 on regulation of secondary metabolism, including FB₁ biosynthesis. Disruption of a hexokinase-encoding gene
324 significantly reduced FB₁ synthesis and osmotic stress tolerance (Kim et al., 2011).

325 Diverse field studies reported the relevance of the dynamic of water activity in maize hybrids for fumonisin
326 contamination in kernels (Battilani et al. 2011; Herrera et al., 2010). In the laboratory temperatures and water
327 activities below 17 °C and 0.94, respectively, have been reported marginal for *F. verticillioides* growth (Marín et al.,
328 2010). In contrast, our results showed an increase in fumonisin accumulation from 60 to 100 days after silking,
329 in which temperature and water activity characteristics were unfavorable for mycelia growth, according to
330 previously published reports. A considerable number of days had mean temperatures below 15 °C and kernel
331 moisture which dropped from approximately 30 to 20 % [corresponding to water activities of approximately 0.96
332 and 0.92, respectively (Maiorano et al., 2010)]. In addition, as the kernels developed, the increase in fumonisin
333 accumulation rate was explained in part by an increased number of days with suboptimal temperatures. In our
334 experiment, the increase in the number of days with suboptimal temperatures for *F. verticillioides* growth (days
335 when the mean temperature between 15 and 20 °C decreased, and with the mean temperature between 10 and 15
336 °C increased) happened when kernel moisture dropped below 30 % [corresponding to water activities of
337 approximately 0.95-0.97 (Maiorano et al., 2010)]. These results are in accordance with previous *in vitro* studies in
338 which high osmotic stress greatly reduced *F. verticillioides* growth and increased fumonisin biosynthesis, while
339 temperature was less directly related to fumonisin production (Jurado et al., 2008; Samapundo et al., 2005). The
340 effect of temperature on fumonisin production at water activity values optimal for fungal growth was only
341 marginal, whereas at lower water activities the effect of temperature was more pronounced and fumonisin
342 production became higher at temperatures not optimal for growth (Samapundo et al., 2005).

343 In conclusion, the high prevalence of kernel infection by *F. verticillioides* as kernels develop increases the risk of
344 contamination with fumonisins, especially during the kernel drying stages. In this study, levels of fumonisins in
345 kernels harvested 100 days after silking (with approximately 20% kernel moisture) were above allowed levels for
346 human consumption (13.99 and 7.16 µg/g of dry weight in the early and late plantings, respectively) in the EU [4
347 µg/g in unprocessed maize (Commission Regulation 1126/2007)]. Contamination risk began earlier in the late
348 planted trial, but by 100 days after silking the risk was higher in the earlier planted trial. Fumonisin accumulation
349 rate was constant until the kernels reached physiological maturity, after which faster accumulation of fumonisin
350 occurred indicating that factors other than kernel developmental stage, such as local environmental conditions,
351 were influencing fumonisin accumulation in the kernels. Feeding damage to the kernels by the larvae *S. cerealella*
352 was also determined to play a role in fungal growth and, consequently, in fumonisin accumulation. The small
353 deviations of the predicted fumonisin accumulation rate based on fungal growth rate could be explained, in part,
354 by increased stress conditions due to more days with suboptimal temperatures for *F. verticillioides* growth when
355 water activity values were low.

356 Table 1. Means or ranks of ear and kernel traits at each sampling date (20, 40, 60, 80, and 100 days after silking) and planting date ^a.

	Kernel age (days after silking) <i>Early planting</i>					Kernel age (days after silking) <i>Late planting</i>				
	20	40	60	80	100	20	40	60	80	100
<i>MEANS</i>										
Ergosterol ^b	---	0.17 c	0.93 bc	1.14 bc	4.80 a	0.79 c	0.17 c	0.65 c	1.35 bc	2.36 b
Kernel moisture ^c	80 a	49 b	31 c	24 d	20 e	83 a	49 b	32 c	24 d	22 de
<i>S. cerealella</i> damage ^d	---	0.08 e	4.92 d	15.25 b	26.97 a	0.03 e	0.08 e	0.53 e	2.31 de	9.37 c
<i>F. verticillioides</i> ^e	---	9 d	32 cd	70 ab	63 ab	36 c	11 cd	34 cd	47 b	76 a
<i>F. proliferatum</i>	---	0.00 b	4.70 a	0.34 b	2.26 b	0.00 b	0.00 b	1.37 b	1.41 b	0.00 b
Other <i>Fusarium</i>	---	1 c	12 abc	14 abc	18 ab	4 bc	9 bc	19 ab	27 a	15 abc
Total <i>Fusarium</i> spp.	---	10 d	48 b	84 a	83 a	40 bc	21 cd	54 b	76 ab	91 a
<i>Penicillium</i>	---	0.44 b	0.22 b	15.33 a	5.78 ab	2.22 b	3.33 b	1.56 b	1.33 b	2.00b
<i>Aspergillus</i>	---	0.22b	4.00 a	0.00 b	0.00 b	0.00 b	0.22 b	0.00 b	0.00 b	0.22 b
Total Molds	---	19 d	61 b	95 a	88 a	46 cd	30 d	60 bc	88 a	97 a
FB ₁ ^f	0.004 c	0.04 c	0.16 c	0.14 c	0.94 a	0.003 c	0.04 c	0.12 c	0.45 b	0.65 b
FB ₂	0.05 cd	0.09 cd	0.12 cd	0.13 c	0.46 a	0.03 d	0.10 cd	0.14 c	0.26 b	0.33 b
Total FB	0.05 cd	0.12 cd	0.25 c	0.24 c	1.02 a	0.04 d	0.13 cd	0.23 cd	0.55 b	0.74 b
<i>RANKS</i>										
Husk tightness ^g	0.58	0.58	0.46	0.46	0.41	0.67	0.55	0.45	0.41	0.41

	(0.43-0.71)	(0.43-0.70)	(0.37-0.55)	(0.37-0.55)	(0.35-0.47)	(0.49-0.79)	(0.39-0.70)	(0.33-0.58)	(0.29-0.53)	(0.29-0.53)
Borer damage	0.54	0.64	0.47	0.42	0.42	0.87	0.50	0.31	0.29	0.52
	(0.33-0.72)	(0.51-0.75)	(0.32-0.61)	(0.28-0.59)	(0.25-0.61)	(0.80-0.89)	(0.37-0.63)	(0.21-0.44)	(0.21-0.42)	(0.37-0.65)
Fusarium ear rot	0.80	0.72	0.37	0.40	0.20	0.80	0.63	0.49	0.30	0.26
	(0.75-0.83)	(0.62-0.79)	(0.24-0.54)	(0.32-0.49)	(0.14-0.32)	(0.75-0.84)	(0.53-0.72)	(0.33-0.65)	(0.20-0.46)	(0.18-0.37)

^aFor each trait, means followed by the same letter did not significantly differ at the 0.05 probability level [Fisher's least significant difference(LSD)].

^bErgosterol units are µg/g of fresh weight; ^c kernel moisture as percentage; ^d damage by *S. cerealella* measured as number of kernel perforated by the moth; ^e molds as percentage of kernels infected; ^fFB₁, FB₂, total FB concentrations were calculated based on fresh weight and then log-transformed; ^ghusk tightness evaluated by a visual scale from 1 (loose husks with visible cob) to 5 (tight husks) (Wiseman and Isenhour, 1992). Ratings for Fusarium ear rot and borer damage were based on a visual rating from 1 (100% of ear totally infected-damaged) to 9 (no infection or damage). Analysis based on rank transformations. Estimated relative effects and confidence interval (95%) for relative treatment effect (lower-upper limit).

363 Table 2. Simple coefficients of correlation among traits recorded in maize ear and kernel samples collected at different kernel development stages (20, 40, 60, 80,
364 and 100 days after silking) at each planting date (n=10).

	Kernel age	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
Ergosterol (1) ^a	0.76 *														
Husk tightness (2) ^b	-0.82 **	-0.51													
Borer damage (3)	-0.60	-0.32	0.77 **												
Kernel moisture (4)	-0.92 **	-0.51	0.87 **	0.70 *											
<i>S. cerealella</i> damage (5)	0.74 *	0.91 **	-0.60	-0.36	-0.57										
Fusarium ear rot (6)	-0.95 **	-0.78 *	0.84 **	0.63	0.90 **	-0.72 *									
Total <i>Fusarium</i> spp. (7) ^c	0.87 **	0.70 *	-0.62	-0.51	-0.68 *	0.68 *	-0.83 **								
<i>F. verticillioides</i> (8)	0.81 **	0.69 *	-0.51	-0.31	-0.56	0.71 *	-0.72 *	0.97 **							
<i>F. proliferatum</i> (9)	0.21	0.24	-0.49	-0.36	-0.35	0.20	-0.50	0.12	-0.03						
Other <i>Fusarium</i> (10)	0.70 *	0.43	-0.62	-0.84 **	-0.72 *	0.30	-0.77 *	0.70 *	0.50	0.32					
<i>Penicillium</i> (11)	0.32	0.24	-0.26	-0.23	-0.28	0.58	-0.17	0.46	0.55	-0.28	0.05				
<i>Aspergillus</i> (12)	-0.07	-0.14	-0.25	0.01	-0.10	-0.09	-0.16	-0.14	-0.19	0.82 **	-0.10	-0.29			
FB ₁ (13) ^d	0.85 **	0.94 **	-0.60	-0.41	-0.66 *	0.77 *	-0.86 **	0.72 *	0.67	0.22	0.58	0.06	-0.16		
FB ₂ (14)	0.88 **	0.92 **	-0.65 *	-0.49	-0.72 *	0.77 **	-0.88 **	0.71 *	0.64	0.22	0.62	0.08	-0.17	0.99 **	
Total FB (15)	0.88**	0.93 **	-0.64 *	-0.46	-0.71 *	0.77 *	-0.89 *	0.74 *	0.67 *	0.23	0.62	0.07	-0.15	1.00 **	1.00 **

365 *, ** Significant at the 0.05 and 0.01 probability levels, respectively.

366 ^a Ergosterol units are µg/g of fresh weight.

367 ^b Husk tightness was evaluated by a visual scale from 0 (loose husks with visible cob) to 5 (tight husks), ear rot by a visual rating from 1 (100% of ear totally
368 damaged by the fungus) to 9 (no damage), ear damage by borers on a similar visual rating from 1 (100% of ear totally damaged by borers) to 9 (no damage), kernel
369 moisture measured as percentage, and damage by *S. cerealella* measured as number of kernel per ear perforated by the moth.
370 ^c *Fusarium* infections were presented as percentage of infected kernels.
371 ^d FB₁, FB₂, total FB concentrations were calculated based on fresh weight and then log-transformed.
372

373 Table 3. Climatic variables^a calculated for the 20-day period before each sampling date (20, 40, 60, 80, and 100 days after silking) for each planting date.

	Kernel age (days after silking) <i>Early planting</i>					Kernel age (days after silking) <i>Late planting</i>				
	20	40	60	80	100	20	40	60	80	100
Average T _m (°C)	17.92	19.56	18.81	17.14	15.49	19.55	18.88	17.26	15.91	14.88
Average T _{min} (°C)	12.92	13.17	12.66	11.29	10.79	13.53	12.80	10.78	10.85	11.88
Average T _{max} (°C)	22.60	26.01	25.53	23.82	21.80	25.52	25.42	24.62	22.29	18.65
Relative humidity (%)	81.56	76.59	74.96	82.71	85.37	76.53	77.25	77.59	84.86	91.98
Daily rainfall (P) (mm)	3.60	0.12	0.53	7.68	7.67	0.73	0.54	0.81	9.38	9.47
Days T _{min} ≤15 °C	16	18	18	17	17	18	16	20	16	17
Days T _{max} ≥30 °C	0	2	3	0	0	2	3	0	0	0
Days 10≤T _m <15 °C	0	0	0	3	9	0	0	2	8	9
Days 15≤T _m <20 °C	19	13	15	16	11	14	14	18	11	10
Days 20≤T _m <25 °C	2	7	5	1	0	7	6	0	1	0
Days 25≤T _m <30 °C	5	0	2	6	7	1	2	2	6	12
Days P≥2 mm	5	0	1	6	7	1	2	2	6	12

374 ^a T_m stands for mean daily temperature, T_{min} for minimum daily temperature, T_{max} for maximum daily temperature, relative humidity for mean daily relative
375 humidity, and daily rainfall for mean daily rainfall (P).

376 Table 4. Variability explained and the sign of regression coefficient (between parenthesis) in multiple linear regressions of differentials for infection by *F. verticillioides*
 377 and ergosterol and fumonisin contents between consecutive sampling dates by changes for kernel characteristics, and climatic variables^a calculated for the 20-day
 378 period before kernel sampling dates (20, 40, 60, 80, and 100 days after silking) in two planting dates (n=8).

	Tmax ≥30 °C	10≤ Tm <15 °C	15≤ Tm <20 °C	Daily rainfall (P)	P ≥2 mm	<i>S. cerealella</i> damage ^b	Ergosterol
	days	days	days	mm	days	no.	µg/g
<i>F. verticillioides</i> ^c	0.34(-)	-		-	0.33(+)	-	-
Ergosterol	-	-		-	-	0.49(+)	-
Total FB	-	-	0.06 (-)	-	-	-	0.83(+)
Total FB ^d		0.49(+)		0.40(-)	-	-	-

379 ^a Tm stands for mean daily temperature, Tmax for maximum daily temperature, and daily rainfall for mean daily rainfall (P).

380 ^b Damage by *S. cerealella* measured as number of kernel perforated by the larvae.

381 ^c Percentage of kernels infected by *F. verticillioides*.

382 ^d Ergosterol content was not included in the stepwise model.

383

Figure 1. Graphical representation for ear and kernels traits at each sampling date (20, 40, 60, 80, and 100 days after silking) for three maize hybrids evaluated at two planting dates.

405 Ratings for Fusarium ear rot and borer damage were based on a visual rating from 1 (100% of ear totally infected
406 damaged) to 9 (no infection or damage).

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