International Journal of Food Microbiology

Enviromental 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.

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

1. Introduction

Fusarium verticillioides (Sacc.) Nirenberg can infect maize (*Zea mays* L.) at most stages of the plants 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 from 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 (Bennet 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 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.

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 (42º24' N, 8º38' 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

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 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 35º C for one week and maintained at 4ºC 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 (ºC), average daily maximum temperature (ºC), average daily minimum temperature (ºC), average daily mean relative humidity (%), daily mean precipitation (mm), number of days with minimum temperature ≤ 15 ºC, number of days with maximum temperatures ≥ 30 ºC, number of days with mean temperature ≥ 10 °C and < 15 °C, ≥ 15 and < 20 °C, ≥ 20 and < 25 °C, ≥ 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; Marín 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 ºC 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 ºC 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 et al. 2009). The remaining disinfected 50 kernels were incubated at 25 ºC for six days on Petri dishes containing MGA (Malachite Green Agar) culture medium for isolation of *Fusarium* species (Alborch et al., 2010; Castellá 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 ºC for seven days (12:12 hours 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 ºC for 16-18 hours 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 ºC for 15 days (12:12 hours 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 contained PDA medium and incubated for 7 days at 25 ºC (12:12 hours 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 in a Pulverisette 14 rotor mill (Fritsch GmbH, Oberstein, Germany). For ergosterol analysis, 50 ml of methanol (HPLC grade) were added to 10 g of maize flour; the mixture was shaken for 30 minutes and then filtered through a sieve of filter paper. Ten ml of the filtered solution was mixed with 1.2 g of potassium hydroxide. Ergosterol extraction was performed twice with 10 ml of hexane in a water bath at 55-60 °C for 30 minutes. The upper layers were recovered, combined and evaporated in a rotary evaporator at 40°C. Extracts were dissolved in methanol (HPLC grade), transferred to a vial and evaporated under a gentle N_2 flow in a sample concentrator (Stuart, Bibby Scientific Limited, Staffordshire, UK). Samples were dissolved in 1 ml of methanol (HPLC grade) prior to HPLC analysis.

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

Fumonisin extraction from the 10 g samples was made with a solvent of 50 ml of distilled water: methanol: acetonitrile (50:25:25) and 1 g of sodium chloride. The mixture was agitated for 20 minutes and filtered through a sieve of filter paper. Ten milliliters of the filtered solution were suspended on 40 ml of PBS. The resulting 50 ml were passed through an immunoaffinity column (Fumoniprep, R-Biopharm Rhône Ltd, UK) and fumonisins were recovered using 1.5 ml of methanol and 1.5 ml of MiliQ water. Fumonisin quantification was performed in a Waters HPLC-system (Waters 2695, separations module, Waters Corporation, Milford, USA) equipped with fluorescence detector (Waters Multi λ Fluorescence Detector 2475, excitation λ at 335 nm and emission λ at 440 nm) and a C18 column (Waters Spherisorb ODS2, 150 mm x 4.6 mm, 5 µm) connected to a precolumn. One hundred µl were injected into the HPLC system after derivatization of fumonisins with *o*-phthaldialdehyde, at 30 ºC and a flow rate of 1 ml/min. The mobile phase was methanol: 0.1 M sodium dihydrogen phosphate (77:23). Quantification was performed using external calibration with FB_1 and FB_2 standard solutions (Sigma, St. Louis, MO, USA), ranging from 0.08 to 2.5 µg/ml. Results were converted into µg/g of dry maize flour. Detection limits for FB_1 and FB_2 were 0.02µg/g and 0.08 µg/g, respectively.

Ergosterol, FB_1 and FB_2 concentrations in a fresh sample were calculated by multiplying the concentrations in 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). Fumonisins were
217 detected in kernels at 20 days after silking and significantly increased with sampling dates (Table 1). Husk

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

The interaction between planting date x sampling (or kernel development stage) was significant for FB_1, FB_2, total FB, borer and Angoumois grain moth damage, and percentage of kernel infected by *Aspergillus* (Table 1). Total fumonisin content were similar until 60 days after silking for both planting dates; however, significant increase in fumonisins occurred 60 to 80 days after silking in the late planting trial and 80 to 100 days after silking in the earlier planted trial. Nevertheless, fumonisin contents in kernels 100 days after silking were significantly higher in the early than in the late planted trial. For this study, fumonisin data were based on kernel fresh weight to minimize kernel weight changes with time, but food and feed safety levels are always based on dry matter content. Thus, our fumonisin concentrations when based on dry matter content were quite high with levels of 13.99 μg/g and 7.16 μg/g at 100 days after planting for the early and late trial, respectively. Kernel damage by *S. cerealella* increased with kernel development and drying in both planting trials but was significantly higher for all sampling dates in the earlier planted trial. Damage to the ears by corn borers was higher in the late planting at increasing kernels age (Table 1).

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

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

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

254 **4. Discussion**

255 *4.1. Fungal infection*

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

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

271 *4.2. Fungal growth*

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

(Samapundo et al., 2005). Kim et al. (2011) reported that enzymes implicated in sugar sensing/signaling networks for controlling growth and development in response to the changing environment also have an important role on regulation of secondary metabolism, including FB_1 biosynthesis. Disruption of a hexokinase-encoding gene significantly reduced FB_1 synthesis and osmotic stress tolerance (Kim et al., 2011).

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

In conclusion, the high prevalence of kernel infection by *F. verticillioides* as kernels develop increases the risk of contamination with fumonisins, especially during the kernel drying stages. In this study, levels of fumonisins in kernels harvested 100 days after silking (with approximately 20% kernel moisture) were above allowed levels for human consumption (13.99 and 7.16 μg/g of dry weight in the early and late plantings, respectively) in the EU [4 μg/g in unprocessed maize (Commission Regulation 1126/2007)]. Contamination risk began earlier in the late planted trial, but by 100 days after silking the risk was higher in the earlier planted trial. Fumonisin accumulation rate was constant until the kernels reached physiological maturity, after which faster accumulation of fumonisin occurred indicating that factors other than kernel developmental stage, such as local environmental conditions, were influencing fumonisin accumulation in the kernels. Feeding damage to the kernels by the larvae *S. cerealella* was also determined to play a role in fungal growth and, consequently, in fumonisin accumulation. The small deviations of the predicted fumonisin accumulation rate based on fungal growth rate could be explained, in part, by increased stress conditions due to more days with suboptimal temperatures for *F. verticillioides* growth when 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) *Early planting*					Kernel age (days after silking) *Late planting*				
	20	40	60	80	100	20	40	60	80	100
MEANS										
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
S. cerealella 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
F. verticillioides [e]	---	9 d	32 cd	70 ab	63 ab	36 c	11 cd	34 cd	47 b	76 a
F. proliferatum	---	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 *Fusarium*	---	1 c	12 abc	14 abc	18 ab	4 bc	9 bc	19 ab	27 a	15 abc
Total *Fusarium* spp.	---	10 d	48 b	84 a	83 a	40 bc	21 cd	54 b	76 ab	91 a
Penicillium	---	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
Aspergillus	---	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
RANKS										
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)

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

[b] Ergosterol 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; [f] FB_1, FB_2, total FB concentrations were calculated based on fresh weight and then log-transformed; [g] husk 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 *											
S. cerealella 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 *Fusarium* spp. (7) [c]	0.87 **	0.70 *	-0.62	-0.51	-0.68 *	0.68 *	-0.83 **								
F. verticillioides (8)	0.81 **	0.69 *	-0.51	-0.31	-0.56	0.71 *	-0.72 *	0.97 **							
F. proliferatum (9)	0.21	0.24	-0.49	-0.36	-0.35	0.20	-0.50	0.12	-0.03						
Other *Fusarium* (10)	0.70 *	0.43	-0.62	-0.84 **	-0.72 *	0.30	-0.77 *	0.70 *	0.50	0.32					
Penicillium (11)	0.32	0.24	-0.26	-0.23	-0.28	0.58	-0.17	0.46	0.55	-0.28	0.05				
Aspergillus (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.

[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 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 moisture measured as percentage, and damage by *S. cerealella* measured as number of kernel per ear perforated by the moth.

[c] *Fusarium* infections were presented as percentage of infected kernels.

[d] FB_1, FB_2, total FB concentrations were calculated based on fresh weight and then log-transformed.

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) *Early planting*					Kernel age (days after silking) *Late planting*				
	20	40	60	80	100	20	40	60	80	100
Average Tm (ºC)	17.92	19.56	18.81	17.14	15.49	19.55	18.88	17.26	15.91	14.88
Average Tmin (ºC)	12.92	13.17	12.66	11.29	10.79	13.53	12.80	10.78	10.85	11.88
Average Tmax (ºC)	22.60	26.01	25.53	23.82	21.80	25.52	25.42	24.62	22.29	18.65
Relative hunidity (%)	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 Tmin ≤15 ºC	16	18	18	17	17	18	16	20	16	17
Days Tmax ≥30 ºC	0	2	3	0	0	2	3	0	0	0
Days 10≤Tm<15 ºC	0	0	0	3	9	0	0	2	8	9
Days15≤Tm<20 ºC	19	13	15	16	11	14	14	18	11	10
Days 20≤Tm<25 ºC	2	7	5	1	0	7	6	0	1	0
Days 25≤Tm<30 ºC	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] Tm stands for mean daily temperature, Tmin for minimum daily temperature, Tmax 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 ºC	10≤ Tm <15 ºC	15≤ Tm <20 ºC	Daily rainfall (P)	P ≥2 mm	*S. cerealella* damage[b]	Ergosterol
	days	days	days	mm	days	no.	μg/g
F. verticillioides[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).

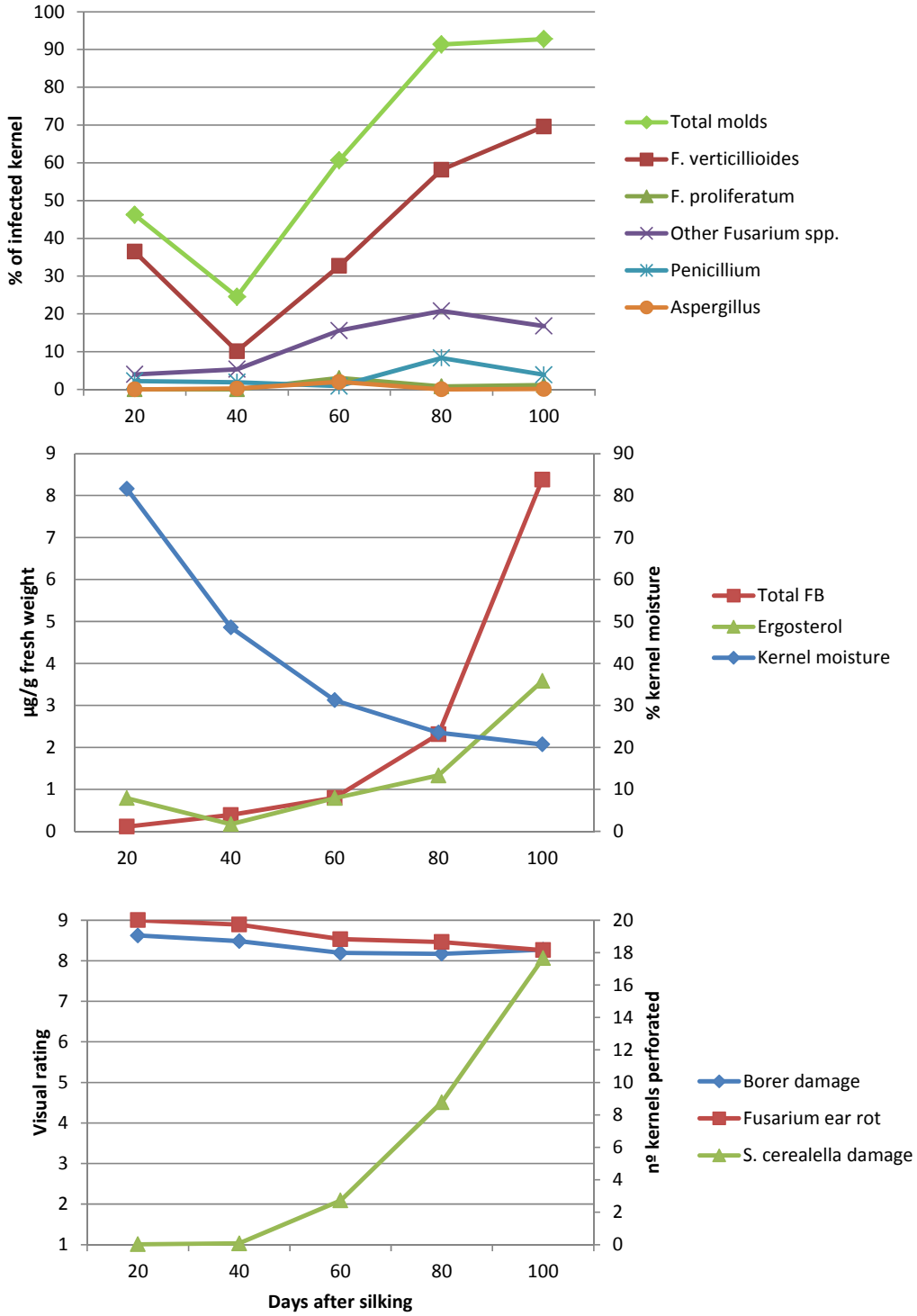
380 [b] Damage by *S. cerealella* measured as number of kernel perforated by the larvae.

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.



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).

Acknowledgements

This research was supported by the National Plan for Research and Development of Spain (AGL2009-12770). A. Cao acknowledges funding from the JAE Program of the Spanish Council of Research. R. Santiago acknowledges postdoctoral contract "Isidro Parga Pondal" supported by the Autonomous Government of Galicia and the European Social Fund.

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