European Journal of Plant Pathology

Assessing white maize resistance to fumonisin contamination

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Abstract

Genetic improvement is an emerging method to reduce the levels of fumonisin (FB) contamination in maize, but breeding advances depend on the development of suitable methods to accurately assess the performance of different cultivars. Our study focused on characterizing a local isolate of *Fusarium verticillioides*; comparing artificial inoculation techniques with this isolate (injection into kernels and down the silk channel); and judging comparing white maize resistance under artificial *vs.* natural inoculation. The fungal growth rate significantly increased with temperature and a_w. The optimum growth rate, corresponding with the shortest phase of initial growth, occurred at 25–30°C and 0.99 a_w. Under silk inoculation with this isolate, the hybrid EP10 × EC22 accumulated significantly less FBs than the other hybrids, whereas, under kernel inoculation, differences among hybrids were not significant ($P \leq 0.05$). The local isolate of *F. verticillioides* produces FBs and responded to the usual environmental conditions during maize kernel ripening in northwestern Spain. Inoculation with this isolate is recommended because it was aggressive, toxigenic, and adapted to the local environment. Silk inoculation was the only method that allowed a clear distinction among genotypes based on differences in resistance to FB accumulation. Resistance to natural and artificial inoculations was confirmed for the hybrid EP10 × EC22.

Key words: *Fusarium verticillioides*; fumonisin; artificial inoculation; maize silks

Introduction

Fusarium verticillioides occurs on plants around the world. Most of its isolates produce fumonisins (FBs), a group of mycotoxins that disrupt the biosynthesis of sphingolipids, which are main components of the plasma membrane of cells, thus adversely affecting human and animal health (Voss et al. 2007; Bennett and Klich 2003; Logrieco at al. 2003). Fumonisin B_1 (FB_1) is the most common and economically important fumonisin, followed by FB_2 and FB_3. Maize is the crop most commonly-contaminated by *F. verticillioides*, and FBs are the most common mycotoxins in maize, although these toxins can occur in other crops as well (CAST, 2003).

Genetic improvement is an emerging method to minimize fungal growth and, therefore, the levels of FBs in maize. Assessing the sources of resistance for breeding purposes depends on the availability of appropriate inoculation techniques that factor in the type and amount of inoculum, as well as inoculation timing and techniques (Eller at al. 2008). A single aggressive and toxigenic isolate of *F. verticillioides* could be used to screen for both resistance to Fusarium ear rot and FB accumulation, because no *F. verticillioides* isolate × maize genotype interaction occurred for Fusarium ear rot severity and FB concentration, and maize resistance should be fairly stable irrespective of the composition of the pathogen population (Reid et al. 1993; Miedaner et al. 2010). With respect to inoculation timing, repeatable results for superior lines have been reported when silk and kernel inoculations were performed 7 d after mid-silks (Mesterhazy et al. 2012). More controversy exists about the best artificial inoculation technique to be applied.

The ideal inoculation technique must result in a sufficient level of infection to differentiate among genotypes for resistance but be below the infection threshold at which differences become difficult to observe (Mesterhazy et al. 2012). The inoculation

techniques most often tested include inserting a *Fusarium*-colonized toothpick into the ear or the silk channel, pinbar inoculation, spraying a spore suspension onto silks, and injecting a spore suspension down the silk channel or through the ear husks into the kernels (Mesterhazy et al. 2012; Munkvold et al. 1997; Bush 2001; Clements et al. 2003). Of these techniques, injections in kernels or silk channel more efficiently distinguished among genotypes resistant to Fusarium ear rot and FB concentration and appear to be the best alternatives for artificial inoculation with *F. verticillioides* (Eller et al. 2008; Bush 2001; Clements et al. 2003). In Ontario (Canada), Schaasfsma et al. (2006) found a consistent correlation between Fusarium ear rot and FB accumulation after kernel-wounded inoculation with *F. verticillioides,* but not with silk channel inoculation, suggesting that the former was more suitable when the goal is to screen genotypes for their resistance to FB accumulation using Fusarium ear rot as an indirect selection criterion. However, other studies carried out in the USA and Germany using silk channel inoculation showed high and significant genetic correlations, ranging from 0.76 to 1.00, between FB content and Fusarium ear rot severity (Robertson et al. 2006; Löffler et al. 2010).

Mesterházy et al. (2012) pointed out that data from natural and artificial silk channel inoculation were highly correlated because silks seemed to be the most important pathway for *F. verticillioides* infection. However, depending on the environment, *F. verticillioides* may use other ways to enter the kernel that must be considered (Munkvold et al. 1997; Bush 2001). Kernel and silk channel inoculations mimic infection vectored by insects and by spores deposited on silks by rain and/or wind, respectively. Therefore, it is not surprising that the effectiveness of these inoculation techniques in discriminating resistant and susceptible hybrids depends on the environment. Natural environmental conditions could favor different ways of kernel

colonization by *F. verticillioides*, through pericarps damaged by insects or through the open stylar canal of unwounded kernels (Eller et al. 2008; Bush 2001; Duncan and Howard 2010).

In Canada and the USA, several studies have been carried out to compare different inoculation methodologies (reviewed by Mesterházy et al. 2012). However, to our knowledge, little research on inoculation techniques has been done in Europe, although such studies are necessary to determine the best methods for assessing maize resistance in each region of Europe. This study was done along the southern Atlantic European Coast, which has a peculiar climate that differs distinctly from Northeastern American climates. Summer temperatures average around 25°C, the other seasons are mild, and the region experiences high rainfall (900–2000 mm a year), particularly from fall to spring. In addition, previous studies focused on yellow varieties of maize, whereas the maize genotypes used in the current research were white maize hybrids, because food safety concerns emphasize this type of maize, and it is preferred worldwide for direct human consumption. White maize is traditionally ground and used to make bread and other bakery products in the northwest region of the Iberian peninsula of Spain (Butron et al. 2009).

Because our ultimate goal is to identify genotypes resistant to fumonisin accumulation in a particular environment, inoculation with local isolates is preferred to using strains from collections, because high variability in both fumonisin content and growth rate has been detected among isolates from different geographic origins (Stepien et al. 2011; Pildain et al. 2004). Temperature must be considered as an environmental factor that influences spore and mycotoxin production under field conditions, in addition to water potential (Indira and Muthusubramanian 2004). In brief, the main goals of our study were to: (i) characterize a local isolate of *F. verticillioides*, (ii)

compare two artificial inoculation techniques with *F. verticillioides* (injection of a spore suspension down the silk channel and into the kernels), and (iii) compare white maize genotype resistance under artificial *vs.* natural inoculation.

Materials and methods

Fusarium verticillioides isolate

The fungal isolate used in this study was an aggressive local isolate of *F. verticillioides* that had been previously isolated from a maize ear (Santiago et al. 2013). This isolate is deposited in the Culture Collection of the Misión Biológica de Galicia (CSIC-Spain) as MBG-1.

Incubation, growth measurement, and fumonisin production

The basic medium used in this study was a 3% maize meal extract agar (MMEA) made by boiling 30 g dry maize meal in 1 L water for 30 min and filtering the resultant mixture through two layers of muslin. The volume was brought to 1 L and 1.5% agar was added. The water activity (a_w) of this basal medium was modified by adding known amounts of the non-ionic solute glycerol to obtain an a_w series (0.99, 0.96, 0.94, 0.92 and 0.90 a_w) (Marín et al. 1995). The a_w of representative samples of each medium was checked with an AquaLab Series 3 (Decagon Devices, Pullman, WA, USA).

Petri dishes with MMEA were centrally inoculated with a needle dipped in a spore suspension of the *F. verticillioides* isolate (10^6 spores/mL) harvested from potato dextrose agar sub-cultures. Inoculated Petri dishes of the same a_w were sealed in polyethylene bags to maintain a constant a_w. Three replicate plates per treatment were used and incubated for 42 d in the dark at 5, 10, 15, 20, 25 and 30°C. Two perpendicular diameters of the growing colonies were measured daily (in mm) until the colony reached the edge of the plate or until the incubation period was completed.

The ability of this *F. verticillioides* isolate to produce FBs was also studied under different water activities (0.94, 0.96, and 0.99 a_w) and temperatures (in the dark under constant temperature of 15, 20 and 25°C, and at 12:12 h light-dark intervals at 25:15°C and 15:10°C). In accordance to daily variations, growth rates were only measured in the

light-dark intervals plates in this experiment. Three Petri dishes were used for each temperature–water activity combination and incubated for 14 d. FB production was determined by HPLC after 7 and 14 d of incubation using the plug technique described by Bragulat et al. (2001).

Field experiments under artificial inoculation

Four white maize hybrids were chosen based on their different behavior in a previous study regarding kernel FB accumulation under natural inoculation (Butrón et al. 2006). Among 10 hybrids, EP10 × EC22 and EP65 × EP10 had the lowest FB accumulation and EP71 × EC22 and EP65 × EP71 the highest. The pedigree, kernel type, and source of inbred lines for these crosses can be found in Butrón et al. (2006).

Three adjacent trials were carried out in 2008 and 2009 in Pontevedra, Spain (42° 24′ N, 8° 38′ W, 20 m above sea level). In the first trial, hybrids were inoculated by injecting a spore suspension of the *F. verticillioides* isolate into the silk channel; in the second one, kernel inoculation with *F. verticillioides* was performed; and, in the third one, three different control treatments were assayed: no inoculation, injection of distilled water into the silk channel, and injection of distilled water into kernels. Hybrids were arranged in completely randomized blocks with three replicates for trials inoculated with *F. verticillioides*, and hybrids and treatments were arranged in a split-plot design with three replicates in the control trial. Hybrids were assigned to main plots and treatments to subplots. Each plot, for trials inoculated with *F. verticillioides,* and each subplot, for the control trial, corresponded to a single row of 13 two-kernel hills. Hills within the row were separated by 0.21 m and rows were spaced 0.80 m from each other. At thinning, one plant per hill remained, for a final density of 60,000 plants/ha.

In the *F. verticillioides* inoculated trials, 7 d after silking, 10 primary ears per plot were inoculated with 2 mL of a spore suspension of the local toxigenic isolate of *F.*

verticillioides that had been ecophysiologically characterized. The spore suspension contained 2.5×10^5 and 2.5×10^6 spores per mL in 2008 and 2009, respectively, and was prepared following the protocol established by Reid et al. (1996) with some modifications. Inoculum was injected into the silk channel using a needle (silk channel inoculation trial) or into the center of the ear using a four-needle vaccinator that perforated the husks and injured 3–4 kernels (kernel inoculation trial). In control trials, inoculations were performed as previously described but using 2 mL of distilled water instead of *F. verticillioides* inoculum.

Each year, the three field trials were simultaneously collected. In each plot or subplot, traits were recorded on the 10 ears previously treated (inoculated with *F. verticillioides* or with distilled water into the silk channel or into the kernels), except for uninoculated subplots. At harvest, the following traits were recorded: (1) husk coverage, evaluated on a visual scale from 1 (loose husks with visible cob) to 5 (tight husks) (Wiseman and Isenhour 1992); (2) visual ratings for kernel, cob, and shank damage by corn borers on a nine-point scale (1 > 90% damaged, 2 = 81 to 90% damaged, 3 = 71 to 80% damaged, 4 = 61 to 70% damaged, 5 = 41 to 60% damaged, 6 = 31 to 40% damaged, 7 = 21 to 30% damaged, 8 = 1 to 20% damaged, and 9 = no damage); (3) stem tunneling by corn borers (in cm); (4) kernel moisture (g of water in 100 g of kernels); (5) number of kernels per ear perforated by *Sitotroga cerealella*.

The collected ears were dried at 35°C for a week then hand shelled, and kernel samples were maintained at 4°C and 50% relative humidity until chemical analyses.

Ergosterol and fumonisin quantification

Ergosterol is a sterol exclusively found in fungal cell membranes. Ergosterol and FB determinations in each plot or subplot, depending on the trial, were performed on 10 g of dried ground kernels taken from a representative dried ground kernel subsample of

200 g. Kernels were ground through a 0.75 mm screen in a Pulverisette 14 rotor mill (Fritsch GmbH, Oberstein, Germany). Ergosterol analyses were carried out as per Reid et al. (1999), with slight modifications. Silk tissue (100 mg) was placed in culture tubes along with 2 mL of methanol and 0.5 mL of 2 M NaOH. Tubes were tightly closed with Teflon-lined caps, placed inside capped 1 L plastic bottles, and irradiated in a microwave oven (Teka, model MW-219, Santander, Spain) at 80% power (2450 MHz, 800 W maximum output) for 20 s and, after approximately 5 min, for an additional 20 s. After cooling, samples were neutralized with 1 M aqueous HCl and treated with 2 mL of methanol. The samples were partitioned with 3 × 4 mL of pentane, and the extracted pentane supernatants were combined and evaporated in a rotary evaporator at 50°C. The extracts were then redissolved in 0.5 mL of HPLC-grade methanol and passed through a 13 mm nylon syringe filter (0.45 µm pore size) into 2 mL HPLC vials, and stored at –20°C until HPLC analysis.

Ergosterol was quantified with a Shimadzu HPLC-system (Kyoto, Japan) equipped with a diode array detector. HPLC separation was carried out at room temperature by injecting a 50-µL sample onto an ACE C18 column (150 × 4 mm i.d., 5 µm particle size) at a flow rate of 2 mL/min with acetonitrile-methanol (90–10%) as the eluent under isocratic conditions. The retention time of ergosterol was approximately 8 min. The peak area at the absorption maximum of 282 nm was used for quantification with an external standard obtained from Sigma (St. Louis, MO, USA).

FBs were extracted from 10 g of milled maize using 1 g of NaCl in 50 mL of distilled water: methanol: acetonitrile (50:25:25) as solvent. The mixture was agitated for 20 min and filtered through a sieve of filter paper. Filtered solution (10 mL) was suspended in 40 mL of phosphate buffered saline and the resulting 50 mL were passed through an immunoaffinity column (Fumoniprep, R-Biopharm Rhône Ltd, Glasgow,

UK). FBs were recovered sequentially using 1.5 mL of methanol and 1.5 mL of Milli-Q water.

FBs were quantified in a Waters HPLC-system (Waters 2695, separations module, Waters Corp., Milford, MA, 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 × 4.6 mm, 5 µm) connected to a pre-column (Waters Spherisorb S5ODS2, 10 mm × 4.6 mm i. d.). A volume of 100 µL was injected into the HPLC system after derivatization of FBs with the *o*-phthaldialdehyde reagent prepared according to Shephard et al. (1990). The samples were injected into the HPLC system within 1 min after derivatization at a flow rate of 1 mL/min and at 30°C. The mobile phase was methanol: 0.1 M NaH_2PO_4 (77:23). Quantification was performed using external calibration with FB_1 and FB_2 standard solutions (Sigma), 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 0.02 µg/g and 0.08 µg/g, respectively.

Statistical analyses

The lag phase (initial phase prior to rapid growth) and growth rate of the *F. verticillioides* isolate were estimated according to the Baranyi and Roberts' model (1994). Analyses of variance (ANOVA) for the lag phase, growth rate, FB production were conducted; all sources of variation were considered fixed factors. Mean comparisons were made using Fisher´s protected Least Significant Difference (LSD) at a 0.05 probability level.

The ANOVA of treatments within control trials (inoculation with distilled water into the silk channel, into wounded kernels, and no inoculation) showed no significant differences among treatments or their interactions with hybrids and years for Fusarium ear rot and FB and ergosterol contents (data not shown), thus mechanical injuries

caused by artificial inoculation did not interfere with maize performance. Therefore, the un-inoculated treatment was used as control to test the effects of both artificial inoculation techniques on *F. verticillioides* infection and FB accumulation, and field trials were analyzed as a split-plot design. Hybrids and treatments were considered fixed effects, and years, replications, and their interactions random effects. Mean comparisons were made using Fisher´s protected LSD at a 0.05 probability level. Pearson correlation coefficients among traits recorded for each hybrid were computed for each artificial inoculation technique. All analyses were performed with SAS (2008).

Results

Characterization of the *Fusarium verticillioides* isolate

The ANOVA for lag phase and growth rate showed significant differences among temperature, a_w, and the temperature × a_w interaction (data not shown). The growth rate significantly increased with temperature and a_w, while the lag phase significantly decreased (Figure 1). The optimum growth rate, corresponding with the shortest lag phase, occurred at 25–30°C and 0.99 a_w. The lag phase significantly increased at temperatures below 20°C under high a_w and at temperatures below 25°C under low a_w. No growth occurred at 10°C when a_w was below 0.94, nor below 5°C at any a_w.

The ANOVA for FBs production showed significant differences between days and temperatures (Table S1). No differences were detected among a_w, but interactions with this factor were significant. There were no significant differences between incubation conditions after 7 d of incubation, but differences became significant after 14 d (data not shown). Consequently, discussion is based on measurements taken after 14 d of incubation. No fumonisins were detected for the temperature ranges at low a_w values, the fungal growth was reduced under such conditions (Table 1). At high a_w (0.96–0.99), a constant temperature (15, 20, or 25°C) had no significant effect on FB_1 and FB_2 production.

Field experiments under artificial inoculation

The combined ANOVA for ear rot, ergosterol, and FB_1, FB_2, and total fumonisin (total FB) contents of four maize hybrids under three treatments (silk inoculation with *F. verticillioides*, kernel inoculation with *F. verticillioides,* and un-inoculated treatment) did not show significant differences among treatments or hybrids for any trait evaluated. However, the year × treatment interaction was significant for FB_1, FB_2, and total FB, and the year × treatment × hybrid interaction was significant for Fusarium ear rot (Table

S2). Consequently, mean comparisons among treatments were made in each year independently. In 2008, silk inoculation differed significantly from un-inoculated treatment for Fusarium ear rot, and kernel inoculation differed significantly from un-inoculated for total FB content. In 2009, both artificial inoculation treatments differed significantly from the control for Fusarium ear rot and total FB content (Table 2).

Because we were interested in checking the ability of each artificial inoculation treatment to distinguish among genotypes with different levels of resistance to FB accumulation under un-inoculated conditions, ANOVA of hybrids were performed separately for each artificial inoculation method. Under silk inoculation with *F. verticillioides*, differences among hybrids were significant for FB_1 and total FB contents, while under kernel inoculation no significant differences were detected among hybrids for FB_1, FB_2, and total FB contents (Table S3). For kernel inoculation a significant interaction year × hybrids was shown for Fusarium ear rot, ergosterol content and husk coverage, however the environmental effect on hybrids analyzed was a matter of range and data were next combined. Under silk inoculation, the hybrid EP10 × EC22 accumulated significantly less FBs than the others, whereas, under kernel inoculation, differences among hybrids were not significant (Table 3).

Discussion

Characterization of the *Fusarium verticillioides* isolate

The requirements for growth of the local isolate of *F. verticillioides* were similar to those of other isolates previously studied (Marin et al. 2004). Although previous studies recorded an optimum for FB production between 20–30°C, in the current study we did not find a significant effect on FB_1 and FB_2 production at these temperatures in combination with high a_w (0.96–0.99) (Marin et al. 2004). The range of temperatures tested by Marin et al. 2004 was 7-37°C, therefore significant differences among temperatures are more expectable. In our study the limited range of temperatures tested (close to the optimum 20°C) could be responsible of the absence of differences. Moreover, the present research was carried out in agar instead of maize. At low a_w (0.94) FBs production was significantly higher at 15°C, agreeing with previous studies in which marginal temperatures and a_w conditions for *F. verticillioides* growth increased FB production (Samapundo et al. 2005; Marin et al. 1999). Previous studies have shown that osmotic stress highly increased FB biosynthesis, suggesting that environmental conditions leading to water stress should be avoided to diminish the risk of FB contamination of maize by *F. verticillioides* (Samapundo et al. 2005; Jurado et al. 2008; Marin et al. 2010). The local isolate responded to water stress by increasing FB production only when temperatures were marginal for fungal growth, environmental conditions that are common during maize kernel ripening in northwestern Spain.

Cyclical temperature conditions resemble daily temperature fluctuations and, therefore, ecophysiological behavior under these conditions should specifically be studied, as growth and subsequent mycotoxin production seem to differ from those under isothermal conditions (Garcia et al. 2012). Previous studies have shown that cyclical temperatures between 10 and 25°C were more favorable to FB_1 production than

a constant temperature of 25°C (Ryu et al. 1999). In the present study, the effect of temperatures from 15–25°C also depended on a_w, because this interval was optimal for FB production at high a_w but was one of the worst temperature intervals, along with 10–15°C, for FB production at low a_w.

Field experiments under artificial inoculation

Kernel artificial inoculation increased FB contamination compared to the control more than did silk inoculation, but both inoculation techniques were highly effective for accumulating mycotoxin (Schaafsma et al. 1993, 2006). Kernel inoculation is an invasive technique that facilitates the direct entrance of *F. verticillioides* into the kernel, avoiding external barriers to fungal progression, such as silks and husks, while silk inoculation mimics infection by spores deposited on silks by rain and/or wind.

Under silk inoculation, the hybrid EP10 × EC22 accumulated significantly less FBs than the other hybrids, corroborating its partial resistance to FB contamination observed under natural inoculation in a previous study (Butron et al. 2006). However, under kernel inoculation, although the patterns of hybrid performance were similar to those observed under silk inoculation (EP10 × EC22 was generally least and EP65 × EP71 most susceptible to FB accumulation), differences among hybrids were not significant, suggesting that the four hybrids had comparable susceptibility to FB accumulation once *F. verticillioides* has been introduced into the kernel.

Although no significant differences were found among hybrids in terms of husk coverage or cob/shank damages, negative correlations of these traits and the fumonisins content were observed. Significant relationships between total FB ($FB_1 + FB_2$) and husk coverage (r= –0.97), and cob (r= –0.97) and shank damage (r= –0.98) by corn borers were observed under silk inoculation with *F. verticillioides.* Silks are the main pathway for *F. verticillioides* entrance into maize kernels; the hyphae can grow along the outside

of silks and within the stylar tissue before the fungus enters into unwounded kernels via an open stylar canal (Munkvold et al. 1997; Duncan and Howard 2010). Therefore, longer silk channels and/or silks packaged more compactly due to high husk coverage could delay fungal movement through the silk channel, affecting kernel susceptibility to *F. verticillioides* in particular genotypes (Miller et al. 2007; Venturini et al. 2011).

Another pathway for *F. verticillioides* entrance into the developing maize kernel could be through kernel placental cells via the shank and cob of the ear (Miller et al. 2007). The high Pearson correlation coefficient between FBs content and shank and cob damage by corn borers suggested that corn borer attack could play an important role in *F. verticillioides* dispersion. The main corn borer in the Mediterranean area, *Sesamia nonagrioides* (Lef.), preferentially damages the stem and shank of the plant and has been described as an important vector for *F. verticillioides* infection (Velasco et al. 2002, 2007; Avantaggiato et al. 2003). The relationship between kernel damage by *S. nonagrioides* and FB content has been established (Avantaggiato et al. 2003), but under our environmental conditions kernel damage by borers tended to be lower (unpublished data). However, higher resistance to shank and cob attack by *S. nonagrioides* could contribute to reduced FB accumulation when enough inoculum is present.

Conclusions

The local isolate of *F. verticillioides* produces FBs and responded to the usual environmental conditions during maize kernel ripening in northwestern Spain by increasing FB production. Inoculation with this isolate is recommended to test for maize resistance to FB accumulation. Both artificial inoculation methods evaluated here resulted in sufficient FB accumulation, but silk inoculation is recommended because it clearly separated genotypes based on differences in resistance to FB accumulation. Kernel inoculation mimics kernel pericarp damage by insects, but neglects the effects of husk coverage and shank and cob, which affect resistance to *S. nonagrioides* attack, on resistance to FB accumulation; these factors may be crucial for preventing FB contamination in northwestern Spain.

Acknowledgements

This research was supported by the National Plan for Research and Development of Spain (AGL2009-12770), the Autonomous Government of Galicia (PGIDIT06TAL40301PR) and the Excma. Diputación Provincial de Pontevedra. 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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Table 1. Mean FB_1 and FB_2 production (ng/mm^2) and fungal growth rate (mm/day) by a local isolate of *Fusarium verticillioides* after 14 d of incubation on maize meal extract agar at different temperatures and water activities (a_w).

a_w	Temperature (°C)	FB_1	FB_2	Growth rate
0.94	15-10	< dl[1] c	< dl c	0.62 b
	25-15	< dl c	< dl c	2.31 a
	15	13.45 a	< dl c	—
	20	5.48 b	2.04 b	—
	25	4.11 b	2.21 a	—
	LSD	2.16	0.16	0.73
0.96	15-10	< dl b	< dl b	1.90 b
	25-15	9.88 a	3.71 a	5.04 a
	15	6.87 a	2.82 a	—
	20	7.50 a	2.44 a	—
	25	8.11 a	2.74 a	—
	LSD	6.03	1.55	0.46
0.99	15-10	4.99 a	< dl c	4.19 b
	25-15	9.15 a	3.85 a	9.26 a
	15	3.07 a	1.43 b	—
	20	6.39 a	2.30 b	—
	25	7.30 a	1.88 b	—
	LSD	—	1.38	0.38

Means followed by the same letter within a column for a determined a_w did not significantly differ at the 0.05 probability level.

[1] dl: detection limit.

Table 2. Means of three inoculation treatments (kernel inoculation with *F. verticillioides*, silk inoculation with *F. verticillioides*, and no inoculation) across four white maize hybrids for Fusarium ear rot, and ergosterol and fumonisin [FB_1, FB_2 and total FB (FB_1+ FB_2)] contents (µg/g) in 2008 and 2009.

Year	Treatment	Fusarium ear rot[1]	Ergosterol	FB_1	FB_2	Total FB
2008	Kernel inoculation	8.20 a	13.30 a	5.01 a	1.64 a	6.65 a
	Silk inoculation	7.83 b	12.59 a	4.52 a	1.34 ab	5.86 ab
	No inoculation	8.50 a	14.25 a	3.27 a	0.83 b	4.10 b
	LSD	0.32	—	—	0.53	1.84
2009	Kernel inoculation	7.70 b	18.79 a	8.25 a	1.97 a	10.22 a
	Silk inoculation	7.80 b	12.13 a	3.20 b	0.18 b	3.38 b
	No inoculation	8.23 a	7.32 a	2.01 c	0.15 b	2.17 c
	LSD	0.34	—	0.91	0.17	1.01

Means followed by the same letter did not differ at the 0.05 probability level.

[1] Estimated on a 9-point visual scale, 1 > 90% of kernels showing infection symptoms and 9 = no visible infection.

Table 3. Mean comparison of four white maize hybrids under each treatment of inoculation with *F. verticillioides* (silk and kernel artificial inoculations) in 2008 and 2009 for fumonisin [FB_1, FB_2, and total FB (FB_1+ FB_2)] content and traits likely related to fumonisin accumulation.

Treatment	Hybrid	Fusarium ear rot[1]	Ergosterol	FB_1	FB_2	FB total	Husk coverage[2]	Damage by corn borers[1]		
								Kernel	Cob	Shank
		1–9	µg/g	µg/g	µg/g	µg/g	1–5	1–9	1–9	1–9
Silk inoculation	EP10 × EC22	7.55 a	18.92 a	3.41 b	0.69 a	4.10 c	2.83 a	7.99 a	8.69 a	7.16 a
	EP65 × EP10	7.98 a	7.31 a	4.10 a	0.77 a	4.87 ab	1.33 a	7.88 a	6.98 a	5.97 a
	EP65 × EP71	7.94 a	16.98 a	4.14 a	0.92 a	5.06 a	1.00 a	8.04 a	6.46 a	5.43 a
	EP71 × EC22	7.79 a	5.07 a	3.90 a	0.76 a	4.65 b	1.40 a	8.15 a	7.86 a	5.99 a
	LSD	—	—	0.37	—	0.30	—	—	—	—
Kernel inoculation	EP10 × EC22	8.20 a	14.89 a	6.24 a	1.57 a	7.81 a	2.00 a	8.42 a	8.57 a	7.69 a
	EP65 × EP10	7.54 a	26.13 a	6.25 a	1.67 a	7.92 a	1.00 a	7.65 b	7.36 a	6.50 a
	EP65 × EP71	7.95 a	16.99 a	7.14 a	2.06 a	9.20 a	1.00 a	7.84 b	6.88 a	4.65 a
	EP71 × EC22	8.10 a	6.17 a	6.91 a	1.91 a	8.81 a	1.00 a	8.43 a	8.32 a	6.70 a
	LSD	—	—	—	—	—	—	0.56	—	—

Within each column and treatment, means followed by the same letter did not significantly differ at the 0.05 probability level.

[1] Ratings for Fusarium ear rot and kernel, cob, and shank damage by corn borers were based on a 9-point visual from 1 (> 90% damaged) to 9 (no damage)

[2] Husk coverage on a 5-point visual scale from 1 (loose husks with visible cob) to 5 (tight husks).

Figure 1. Growth rate (A) and lag phase (B) of the local isolate of *Fusarium verticillioides* when incubated at different temperature and water activity (a_w) conditions. Columns with the same letter within a determined a_w did not significantly differ at the 0.05 probability level.