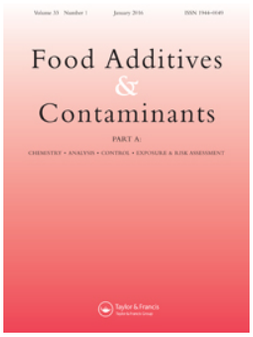

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Ecophysiology of *Fusarium temperatum* isolated from maize in Argentina

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ABSTRACT

The effect of water activity (a_w = 0.95, 0.98 and 0.995), temperature (15, 25 and 30°C), incubation time (7, 14, 21 and 28 days), and their interactions on growth and moniliformin (MON), beauvericin (BEA), fusaproliferin (FUS) and fumonisin B_1 (FB_1) production by two strains of *Fusarium temperatum* isolated from Argentinean maize were determined *in vitro* on sterile layers of maize grains. The results showed that there was a wide range of conditions for growth and mycotoxins production by *F. temperatum*. Both strains were found to grow faster with increasing a_w and at 30°C. In relation to mycotoxin production, the two strains produced more FUS than the other mycotoxins regardless of a_w or temperature evaluated (maximum = 50 000 µg g^{-1}). For FUS, MON and BEA, the maximum levels were observed at 0.98 a_w and 30°C (50 000, 5000 and 2000 µg g^{-1} respectively). The lowest levels for these three mycotoxins were detected at 15°C and 0.95 a_w (1700 and 100 µg g^{-1} for FUS and MON respectively), and at 0.98 a_w (400 µg g^{-1} for BEA). The maximum levels of FB_1 were produced at 15°C and 0.98 a_w (1000 µg g^{-1}). At all a_w and temperatures combinations evaluated there was an increase in toxin concentrations with time incubation. The maximum levels were detected at 21 days. Statistical analyses of a_w, temperature, incubation time, and the two- and three-way interactions between them showed significant effects on mycotoxins production by *F. temperatum*. For its versatility on growth and mycotoxin production, *F. temperatum* represents a toxicological risk for maize in the field and also during grain storage.

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Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops in human and animal diets worldwide, and it is a dietary staple, making up the primary portion of calories consumed in many countries (FAO 2011). Aside from providing nutrients for humans and animals, maize serves as a basic raw material for the production of starch, oil, protein, alcoholic beverages, food sweeteners and fuel. Recent reports indicate that this crop is at the centre of global food security as it may become a critical risk if major producers/exporters of maize worldwide are unable to cover expected demands in other parts of the world due to plant diseases (Wu & Guclu 2012).

The FAO estimates that in 2012 the total world production of maize was 880 million tons (FAO 2012), with the United States, China, Brazil and Argentina harvesting 31%, 24%, 8% and 3% of the total production, respectively. Argentina is the second world exporter after the United States. In particular, it exports maize to many countries all over the world, particularly in Europe, Asia, Africa, America and the Middle East. Maize production currently represents 25% of the four major crops cultivated in Argentina. In 2012/13, Argentina increased its production by 26%, producing a supply of 25 million tons (USDA 2014).

The genus *Fusarium* includes several species that are important pathogens of maize and other cereals, causing root, stem and ear rot, with severe crop yield reduction of economic relevance. In addition, certain isolates are also capable of producing mycotoxins that can be accumulated in infected plants or in stored grains.

Worldwide, food supplies often contain unavoidable contaminants, such as mycotoxins, that adversely affect health and hence are subject to regulations of maximum tolerable levels in food. The estimated losses in maize attributable to the *Fusarium* mycotoxins in the United States alone are about US$2900 million/year (Windels 2000). *Fusarium* species are the most prevalent pathogens of maize, and are widespread in all cereal-growing areas of the world, but there are some

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geographical differences in the natural distribution, as well as of their corresponding mycotoxins, which are influenced primarily by environmental conditions, crop production and storage methods (Doohan et al. 2003; Marín et al. 2004). The main *Fusarium* species isolated from maize are *F. verticillioides*, *F. proliferatum*, *F. subglutinans* and *F. temperatum*. They produce several types of mycotoxins depending on the species and the environmental conditions (Chulze et al. 2000; Battilani et al. 2011; Scauflaire et al. 2011; Fumero et al. 2015).

Fumonisins are long-chain polyhydroxyl alkylamines with two propane tricarboxilic acid moieties esterified to hydroxyls on adjacent carbons. There are four main groups in relation to the molecular structure of fumonisins, identified as fumonisins A, B, C and P. The fumonisin B (FB) analogues, comprising toxicologically important FB_1, FB_2 and FB_3, are the most abundant naturally occurring fumonisins, with FB_1 predominating and usually being found at the highest levels. Apart from the FB series, some of the other analogues may occur in naturally contaminated maize at relatively low levels (5% of the total fumonisins present) (CAST 2003). Fumonisins are produced by *F. verticillioides*, *F. proliferatum*, *F. oxysporum*, *F. globosum* and several other *Fusarium* species, and they are frequently found in corn and corn-based foods. FB_1 is the most commonly found, not only in corn and corn-based foods but also in beer, rice, sorghum, triticale, cowpea seeds, beans, soybeans and asparagus. FB_1 can cause two diseases of farm animals: leucoencephalomalacia in horses and porcine pulmonary oedema. It is also carcinogenic, hepatotoxic, nephrotoxic and embryotoxic in laboratory animals. In humans, fumonisins are associated with oesophageal cancer and neural tube defects; the IARC designated FB_1 in Group 2B as 'possibly carcinogenic to humans'. FB_2 and FB_3 are closely related metabolites that may co-occur in lower concentrations than FB_1 (Scott 2012). Disruption of lipid metabolism appears to be the underling mechanism by which fumonisin cause toxicity by inhibition of ceramide synthase, a key enzyme in the *de novo* sphingolipid biosynthesis pathway. The major biochemical and cellular consequences resulting from blockage of ceramide biosynthesis are the accumulation of sphingoid bases and the global disruption of lipid metabolism (Pitt et al. 2012). Since the principal *Fusarium* mycotoxins in maize are fumonisins, and they are of a great concern because of their multifaceted economic problems related to grain yield reduction or production unfit for sale, and reduced animal productivity due to health problems and human health costs, in Europe and the United States there are legislations that fix maximum tolerated level for total fumonisin in raw maize (4 mg kg^{-1}), in maize derived food for direct human consumption (1 mg kg^{-1}) and in animal feeds destined for pigs and horses (5 mg kg^{-1}) (European Commission 2007).

Fusarium species are also responsible for the production of another group of bioactive compounds called 'emerging' or 'minor' mycotoxins. This group includes beauvericin (BEA), fusaproliferin (FUS) and moniliformin (MON). At present, little information is available about their presence in foods and feeds, and in commodities, but it is known that this group of mycotoxins has acute and chronic toxic effects on human and animal health, such as hepatotoxicity, teratogenicity, carcinogenicity, and reproductive and developmental toxicity (Jestoi 2008).

BEA is a cyclic lactone trimer containing an alternating sequence of three N-methyl L-phenylalanyl and three D-a-hydroxyisovaleryl residues. This toxin is a specific cholesterol acyltransferase inhibitor and can induce apoptosis and DNA fragmentation. Also, BEA increased intracellular calcium by increasing the formation of cation selective channels in the lipid membrane in a line of neuronal cells. The toxin affects the electromechanical and physiological properties of isolated smooth and heart muscle preparations and in *in vitro* assays BEA was toxic to several human cell lines (Jestoi 2008). Reynoso et al. (2004) reported the production of BEA by *F. subglutinans* and *F. proliferatum* isolated from maize in Argentina. Moretti et al. (2007) reported the production of BEA by *F. bulbicola*, *F. denticulatum*, *F. lactis*, *F. phyllophilum*, *F. pseudocircinatum* and *F. succisae*. Scauflaire et al. (2012) and Fumero et al. (2015) showed the production of BEA by *F. temperatum* isolated from maize in Belgium and Argentina, respectively.

FUS is a bicyclic sesterterpene consisting of five isoprenic units; is can form non-covalent interactions between single- and double-stranded DNA-oligonucleotides, which may explain its toxic and teratogenic effects (Jestoi 2008). Moretti et al. (2007) reported the production of FUS by *F. antophilum*, *F. begoniae*, *F. bulbicola*, *F. circinatum*, *F. concentricum*, *F. succisae* and *F. udum*. FUS is also produced by *F. subglutinans and F. temperatum* (Ritieni et al. 1995; Reynoso et al. 2004; Meca et al. 2010; Scauflaire et al. 2012; Fumero et al. 2015). It has been found to be toxic in the brine shrimp (*Artemia salina*) larvae bioassay and mammalian cells and it causes teratogenic effects on chicken embryos (Jestoi 2008).

MON is a sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione. This toxin selectively inhibits mitochondrial pyruvate and α-ketoglutarate oxidations,

thus preventing the entrance of pyruvate and α-ketoglutarate into the tricarboxylic acid cycle with consequent reduction of oxidative phosphorylation, i.e., ATP production. MON is produced at least by 30 *Fusarium* species, isolated from different substrates and geographical areas. In addition to *F. proliferatum*, the *Fusarium* species known to produce significant amounts of MON include *F. avenaceum*, *F. fujikuroi*, *F. nygamai*, *F. pseudonygamai*, *F. subglutinans*, *F. verticillioides* and *F. thapsinum*. MON is a highly toxic metabolite with acute toxicity to plants and extreme toxicity to various animal species. Clinical signs of MON toxicity observed in animals have been generally described as progressive muscular weakness, respiratory distress, cyanosis, coma followed by death (Jonsson et al. 2015). There is scarce information worldwide on the occurrence and contamination levels of these *Fusarium* metabolites in foods and feeds. *F. temperatum* has been reported as a producer of different mycotoxins, such as BEA, FUS, MON and FB_1, but nothing has been considered about the range of environmental conditions that allow this species growth and accumulate these mycotoxins in maize grains. Is important to know the range of conditions over which this species can growth and produce mycotoxins to develop prevention and control strategies.

The objective of this study was to determine the impact of water activity and temperature on the colonisation and production of four different mycotoxins by two Argentinean strains of *F. temperatum* isolated from maize.

Materials and methods

Fungal strains

Two strains of *F. temperatum* isolated from Andean maize harvested in the Northwest (NOA) region of Argentina were used: *F. temperatum* Rio Cuarto, *F. temperatum* (RCFT) 903 and *F. temperatum* RCFT 912. These strains were demonstrated to be FUS, BEA and fumonisin producers by Fumero et al. (2015). Monosporic cultures were cryopreserved in sterile 15% glycerol (Leslie & Summerell 2006). Strains were maintained in the culture collection at the Department of Microbiology and Immunology, National University of Rio Cuarto (UNRC), as RCFT corresponding to *F. temperatum*.

Growth substrate

The strains were grown on irradiated maize grains (12 kGy) adjusted to three different water activities (a_w) adding different volumes of sterile water calculated from a moisture absorption curve for the maize. The maize was equilibrated at 4°C for 72 h and the final a_w levels of each treatment (0.950, 0.980 and 0.995) were confirmed using an Aqualab Series 3.

Inoculation and growth conditions

A 3-mm-diameter agar disk from the margin of a 7-day-old growing colony of each strain of *F. temperatum* grown on Spezieller Nährstoffarmer Agar medium was used to inoculate centrally the Petri dishes with the maize grains. Maize grains with different a_w's (0.95, 0.98 and 0.995) were incubated at temperatures of 15°C, 25°C or 30°C for different incubation periods (7, 14, 21 and 28 days), and radial growth of the colonies was measured daily by taking two diameters at right angles to each other until the colony covered all the plate (10 cm). The diameters (mm) of the colonies were plotted against time (days), and a linear regression was applied to obtain the growth rate (mm day^{-1}) as the slope of the line. The experiment had three replicates per treatment.

Mycotoxin profiles

Three replicates per treatment were destructively sampled after 7, 14, 21 and 28 days, dried in a forced draft oven at 60°C for 2 h, and finely ground and stored at 4°C until the analyses. Samples were analysed for multiple mycotoxins including MON, FUS, BEA and FB_1. For the extraction, a volume of 20 ml of extraction solvent (acetonitrile/water/acetic acid 79:20:1 v/v/v) was added to 5 g of ground substrate and homogenised for 30 min in a rotatory shaker at 150 rpm. The samples were then filtered through Whatman No. 4 filter paper, and an aliquot of 2 ml was transferred into glass vials and the extraction solvent was evaporated totally under N_2 flow. The dried samples were resuspended in 1 ml of methanol and analysed by liquid chromatography with electrospray ionisation triple quadrupole mass spectrometry (LC/ESI-MS/MS) according to Malachováa et al. (2014). The experiment was done with three replicates per treatment.

Statistical analysis

An analysis of variance (ANOVA) test was performed to determine the significance of the effect of each factor and the interactions among them, on the variables analysed, using the software Infostat, with $p < 0.05$. Infostat was also used to determine the growth rates by linear regression. Contour maps and three-

dimensional graphics were done using the software Sigmaplot 10.0.

Results

Effect of water activity and temperature on growth

An ANOVA was separately performed to analyse the effect of the single factors considered in the study (strain, water activity, temperature and incubation time) on growth rate, as well as two- and three-way interactions. All these factors, excluding strain, and their interactions significantly affected growth (Table 1). Figure 1 shows the radial extension rates (mm day^{-1}) of *F. temperatum* RCFT 912 at the different a_w's and temperatures evaluated. Comparison between the two strains showed no differences in growth rates at all a_w's and temperatures evaluated. Temperature affected growth, being the radial extension rates higher at 25°C/30°C than at 15°C. For both strains, optimal growth conditions were 0.980/0.995 a_w and 25°/30°C. At 0.950 a_w, both strains were unable to grow at 15°C, but they could grow at 25 and 30°C. At this low a_w the three temperatures showed significantly different growth rates (15° < 25° < 30°). The two-dimensional contour map on the effect of a_w and temperature on the growth of *F. temperatum* RCFT 912 shows the conditions under which equivalent growth rates occurred under different environmental conditions (Figure 2).

Table 1. Analysis of variance (ANOVA) on the effects of strains, water activity (a_w), temperature (T°) and incubation time, and their interactions on growth of *Fusarium temperatum* on irradiated maize grains.

Source of variation	SS	d.f.	MS	*F*	*P*
Strain	112.04	1	112.04	0.19	0.6656
a_w	14 850.67	2	7425.33	854.15	< 0.0001[a]
T°	24 356.89	2	12 178.45	1400.91	< 0.0001[a]
Incubation time	53 396.99	10	5339.70	614.24	< 0.0001[a]
a_w * T°	2115.01	4	528.75	60.82	< 0.0001[a]
a_w * Incubation time	8064.23	20	403.21	46.38	< 0.0001[a]
T° * Incubation time	12 472.58	20	623.63	71.74	< 0.0001[a]
a_w * T° * Incubation time	1198.82	40	29.97	3.45	< 0.0001[a]
Residual	860.63	99	8.69		
Total	117 315.82	197			

Notes: [a]The factor had a significant effect ($p < 0.05$) according to the ANOVA test.
SS, sum of squares; d.f., degrees of freedom; MS: mean squares, *F*, *F*-value; *P*, *p*-value.

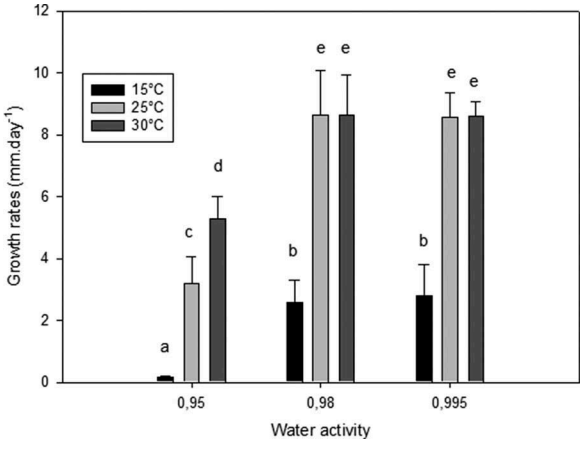

Figure 1. *Fusarium temperatum* RCFT 912 growth rates on irradiated maize grains adjusted to different water activities and growing at three different temperatures. Different letters indicates significant differences (Tukey test, $p < 0.05$).

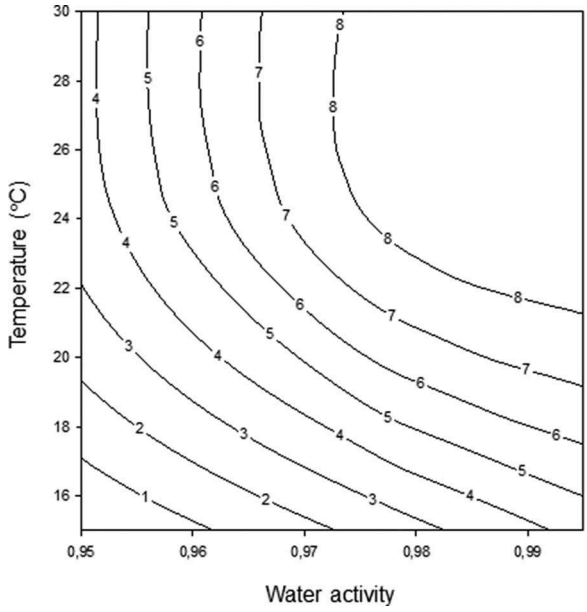

Figure 2. Two-dimensional contour map of growth profile of *Fusarium temperatum* RCFT 912 in relation to temperature and water activity. Numbers on the isopleths refer to similar growth rates (mm day^{-1}).

Effect of water activity and temperature on mycotoxins production

Figure 3 shows the surface response curves for MON, BEA, FUS and FB_1 produced at 15, 25 and 30°C over 28 days of incubation time. The results of this study showed that the time course of production of the toxins evaluated varied with T° and a_w. Statistically, the effects of a_w, T° and incubation time and their interactions were found to be significant, in contrast with the effect of the strain, which was not significant (Table 2). Figure 3 shows that there was an increase in the production of the four toxins with incubation time. The maximum production was reached at 21/28 days, depending on the toxin, and the two strains produced

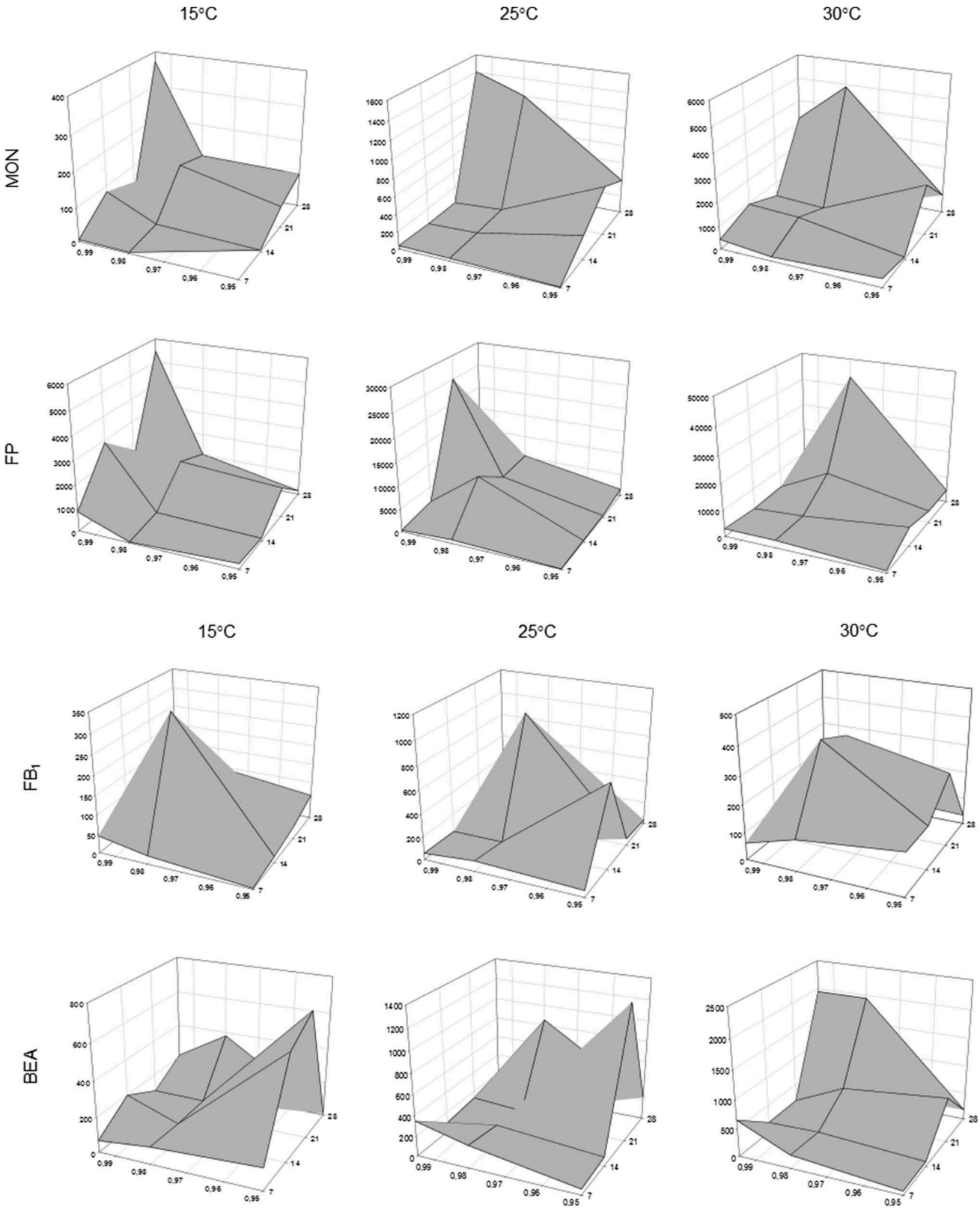

Figure 3. Mycotoxin concentrations (μg g^{-1}) produced by *Fusarium temperatum* RCFT 912 inoculated on irradiated maize grains adjusted at different a_w levels and incubated at 15, 25 and 30°C for 28 days.

more FUS than the other toxins regardless of a_w or temperature. In general, the maximum toxin levels were produced at the optimal conditions for *F. temperatum* growth, except for FB_1, for which the maximum level was detected at 15°C and 0.98 a_w. However, in suboptimal conditions for growth, there

Table 2. Analysis of variance (ANOVA) on the effects of water activity, temperature and incubation time on four mycotoxin production by *Fusarium temperatum* RCFT 912 inoculated on irradiated maize grains.

	MON		BEA		FP		FB_1	
Source of variation	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Strain	0.04	0.8358	0.01	0.9372	1.99	0.1596	2.89	0.0905
a_w	3.99	0.0202[a]	1.43	0.2411	2.01	0.1370	66.78	0.0001[a]
Incubation T°	32.19	0.0001[a]	5.17	0.0065[a]	16.59	0.0001[a]	64.17	0.0001[a]
Incubation time	9.83	0.0001[a]	4.11	0.0075[a]	4.98	0.0024[a]	15.05	0.0001[a]
a_w * Incubation T°	3.17	0.0150[a]	1.48	0.2101	1.89	0.1145	33.55	0.0001[a]
a_w * Incubation time	4.13	0.0007[a]	2.13	0.0519	2.52	0.0231[a]	34.96	0.0001[a]
Incubation T° * Incubation time	3.99	0.0009[a]	1.87	0.0882	2.06	0.0606	35.45	0.0001[a]
a_w * Incubation T° * Incubation time	3.19	0.0004[a]	1.46	0.1422	2.38	0.0070[a]	63.08	0.0001[a]

Notes: [a]The factor had a significant effect ($p < 0.05$), according to the ANOVA test.
BEA, beauvericin; FB_1, fumonisin B_1; FP, fusaproliferin; MON, moniliformin.

was also high production of BEA (0.95 a_w and 25°C), FUS and MON (0.95 a_w and 30°C).

The highest level of MON (7000 μg g^{-1}) was observed at 0.98 a_w and 30°C after 28 days of incubation; as a_w was reduced, MON production declined. Production was lower at 15°C than at 25°C regardless of a_w, and it generally increased with incubation time to 28 days. At 15°C, MON was produced only at 0.995 a_w by *F. temperatum*.

The highest level of BEA (1800 μg g^{-1}) was obtained at 0.98 a_w and 30°C after 28 days of incubation, and at this temperature while a_w was reduced BEA production declined. At 15 and 25°C there were highest BEA levels at 0.95 a_w and increased with temperature. At 15 and 25°C there were also a second maximum of toxin production at 0.98 a_w.

FUS reached the highest levels growing on irradiated maize grains in comparison with the other toxins; the range of production ranged from 1700 to 50 000 μg g^{-1}. The highest level was obtained at 0.98 a_w and 30°C after 28 days of incubation. The production at 15°C was lower than at 25°C and increased with the incubation time to 28 days. At 15 and 25°C the maximum levels were reached at 0.995 a_w. For this toxin there were also peaks of high production at conditions suboptimal for growth: at 0.95 a_w and 30°C, and at 0.995 a_w and 15°C.

The highest FB_1 level (1000 μg g^{-1}) was obtained at 0.98 a_w and 15°C after 21 days of incubation. At this stressful condition for growth, there was another peak of high production at 0.95 a_w (800 μg g^{-1}). This level overcomes the maximum levels reached at 25°C (300 μg g^{-1}) and 30°C (400 μg g^{-1}), both at 0.98 a_w.

Impact of water activity and temperature on mycotoxin profiles

Figure 4 shows the two-dimensional contour plots relating a_w and temperature and their influence on toxin production by *F. temperatum*. This provides a profile of the combined impact of these abiotic factors on mycotoxin production. Both strains of *F. temperatum* evaluated produced the higher levels of MON and FUS at 30°C, but at 0.98 a_w and 0.995 a_w respectively. BEA showed the maximum level at 0.995 and 25°C. Finally, FB_1 was the unique toxin with the maximum levels produced at 15°C and 0.98 a_w.

Analysis of mycotoxin levels on stress conditions

The results showed that some environmental conditions produced lower growth rates for *F. temperatum*; these stress conditions also produced increased toxin production. Figure 1 shows the growth rates of *F. temperatum*; Table 3 shows the stress conditions for growth in which mycotoxins were produced in high levels.

The results of mycotoxin production under stress conditions for fungal growth showed that BEA and FB_1 were detected at high levels at 15°C and 0.95 a_w, regardless of the lower growth rate. BEA was also produced at high levels at the lowest a_w and 25 and 30°C, conditions where the strain had half the maximum growth rate. BEA production could be associated with low water activity stress. FB_1 was also produced at 15°C and 0.98 a_w, this condition being where the maximum level of toxin was produced. Meanwhile, FUS and MON were produced at high levels at 0.995 a_w and 15°C when the growth rate was half the maximum rate.

Discussion

Currently, over 5 billion people worldwide are at risk of chronic exposure to mycotoxin in food, maize being one of the main sources of human exposure to mycotoxins because it is both highly consumed worldwide and is one of the most susceptible crops to *Fusarium* contamination (Wu & Guclu 2010). A knowledge of the ecophysiology of *Fusarium* species and their behaviour under different environmental conditions will permit the prediction of the potential risk of mycotoxins contamination.

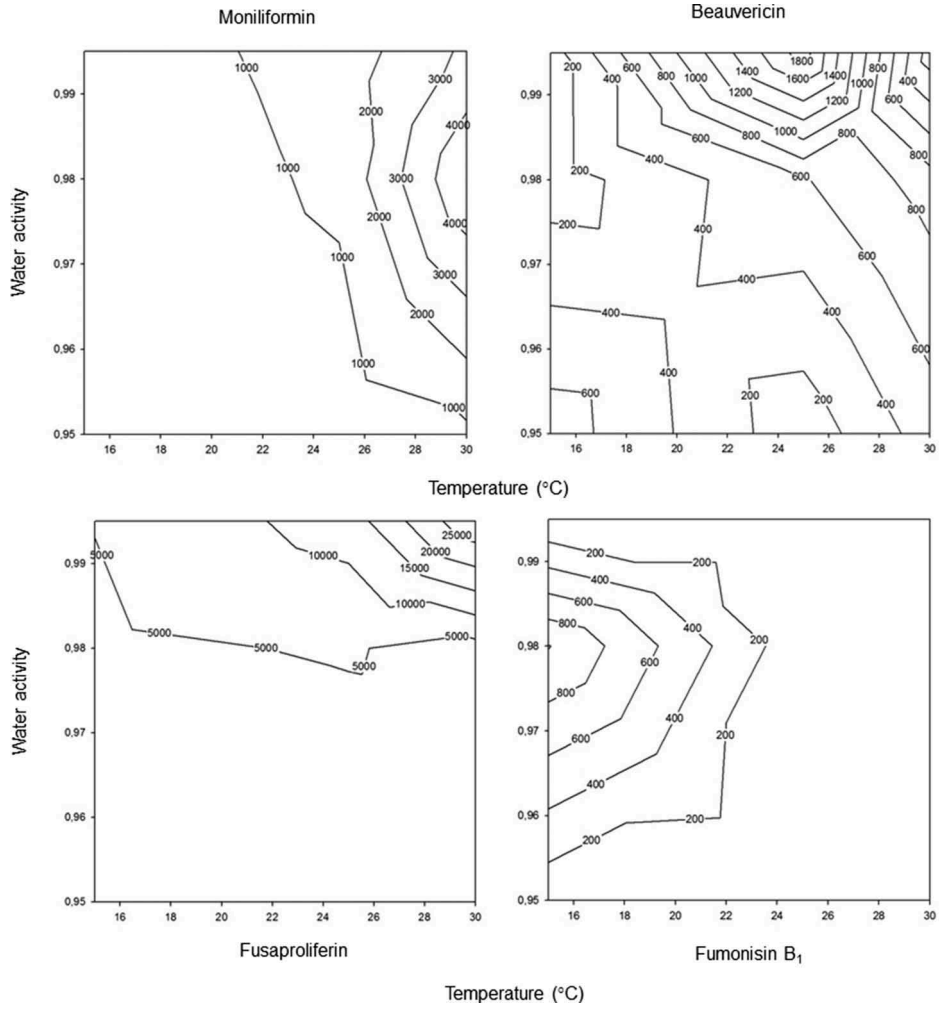

Figure 4. Two-dimensional contour maps of MON, BEA, FUS and FB_1 production at 21 days by *Fusarium temperatum* RCFT 912 in relation to temperature and water activity. Numbers on the isopleths refer to toxin levels (μg g^{-1}).

The effect of environmental conditions on growth and toxin production by *F. temperatum* has not been previously studied in-depth. In the present study, *F. temperatum* strains reached a maximum growth rate at values higher than 22°C, reflecting the local climatic conditions of the regions of isolation of this species. Castellá et al. (1999) showed that the close related species *F. subglutinans* grew optimally at 20–25°C on maize culture media. In concordance with the results observed for other *Fusarium* species, *F. temperatum* showed the lowest growth rates at 15°C and 0.95 a_w. Previous studies used a unique set of conditions (25°C and 40% humidity) to examine the general toxin profile of *F. temperatum*, and production of FUS, BEA, MON and FB_1 was reported (Scauflaire et al. 2012; Stępien et al. 2013; Fumero et al. 2015). In the present study we observed that 25°C and 40% humidity were not necessarily the best conditions for toxin production.

Table 3. Toxin levels produced under stress conditions for fungal growth.

	0.95			0.98			0.995		
	15°C	25°C	30°C	15°C	25°C	30°C	15°C	25°C	30°C
BEA	+	+++	++						
FB_1	+			++					
FP							+		
MON							+		

Note: Shown is the presence of significant levels of each toxin in the stress condition assayed: +, a minor level; ++, an intermediate level; and +++, a higher level of the toxin.
BEA beauvericin, FB_1 fumonisin B_1, FP, fusaproliferin, MON moniliformin.

Major factors affecting the risk of *Fusarium* infection and subsequent mycotoxin contamination are temperature, insect injury, drought stress and water activity (Bush et al. 2004; Wu et al. 2011). At present there is special interest in the infection of maize grains during ripening, post-harvest and storage by *Fusarium* because of their ability to produce several mycotoxins during these crop stages. The ranges of a_w and temperatures used in the present study simulate those occurring in ripening grain and harvest stage (RH = 19–30%; a_w = 0.95–0.995). Fumonisins are the most studied mycotoxins produced

by *Fusarium* in maize. Evidence from several sources indicates that drought stress is associated with elevated levels of *F. verticillioides* infection and fumonisin accumulation in grain (Miller 2001), and it is known that dry periods before or during the grain ripening favour more severe *Fusarium* ear rot and higher levels of fumonisins (Doohan et al. 2003). Low water activity (0.98 a_w) has been proposed to be the more frequent for the FB_1 production by the two main maize and wheat pathogens species, *F. verticillioides* and *F. proliferatum* (Marín et al. 2004; Cendoya et al. 2014), in agreement with our findings with *F. temperatum*. *F. temperatum* produced similar amounts of FB_1 (250 μg g^{-1} at 0.98 a_w and 25°C) compared with *F. verticillioides* growing in maize grain (300 μg g^{-1} at 0.98 a_w and 25°C), but at a lower level than the main producer *F. proliferatum* in maize (2000 μg g^{-1} at 0.98 a_w and 25°C) (Marín et al. 1999). Otherwise, for the main maize pathogen species, *F. verticillioides*, it was reported that the optimal condition for FB_1 production was 30°C and 0.97 a_w, but for *F. proliferatum* the best conditions were 15°C and 0.97 a_w (Marín et al. 1999, 2004; Cendoya et al. 2014). *F. temperatum* produced the maximum levels of FB_1 in conditions of low water activity and temperature, 0.98 a_w and 15°C (1000 μg g^{-1}). In all conditions evaluated, there was an inverse relation between growth and FB_1 production by *F. temperatum*, showing the maximum level at 0.98 a_w and 15°C (1000 μg g^{-1}) when the growth rate was lower than the optimum growth rate (2.5 mm day^{-1}).

F. temperatum was reported to be a producer of emerging toxins (Scauflaire et al. 2012; Stępien et al. 2013; Fumero et al. 2015). Recently, increased importance has been given to the investigation of these mycotoxins in cereals because of their toxic effects on plants and animals (Jestoi 2008). The present work provides the first detailed study of the influence of a_w, temperature and incubation time on the emerging mycotoxin production by *F. temperatum* growing on maize grains.

The natural occurrence of BEA in grains is common worldwide with concentrations ranging from trace levels up to 520 μg g^{-1}; BEA has been found as a natural contaminant of maize harvested in the United States, South Africa and European countries such as Poland, Italy, Switzerland and Slovakia (Jestoi 2008). BEA was recently reported to occur at concentrations of tens of mg kg^{-1} in Southern Europe and Morocco (maximum concentration of 59 mg kg^{-1} in maize from Morocco) (Santini et al. 2012). Meca et al. (2010) reported contamination of cereals available on the Spanish market with BEA and levels varied from 0.5 to 20 μg g^{-1}. In the same studies, however, FUS showed an occasional occurrence up to 19.6 mg kg^{-1} in Morocco, while its natural occurrence in cooler climates seems to be more rare (Meca et al. 2010; Santini et al. 2012). In relation to the presence of this toxin in natural samples, FUS has been determined in maize samples in concentrations up to 500 μg g^{-1} (Ritieni et al. 1997; Pascale et al. 1999). In another study from the United States, Munkvold et al. (1998) reported high contamination levels of animal feeds with FUS, with levels up to 30 μg g^{-1}. MON has been reported as a natural contaminant in maize and other raw cereals (rice, oats, rye, wheat, barley and triticale), and in food and feed, but MON contamination is higher in maize. The presence of MON has been recently studied in Europe, where 23 different cereal samples were analysed for their MON content (Walburga von Bargen et al. 2012). Twenty of them showed positive results with levels up to 126 μg kg^{-1}. In another study in Europe, MON was detected in 52% of 50 poultry feed samples from Slovakia at concentrations from 42 to 1214 μg kg^{-1} (Labuda et al. 2005).

On the basis of our results, the production of BEA by *F. temperatum* could be expected to occur at relatively high humidity and hot conditions because the greatest amounts of this toxin (2000 μg g^{-1}) were observed at 30°C and 0.98–0.995 a_w.

The present study also showed that FUS was the principal toxin produced by *F. temperatum*. The maximum amount reached 100 mg g^{-1} and it was observed at 30°C and 0.98 a_w. In comparison with data about the production of FUS by the related species *F. subglutinans*, the maximum production of FUS in maize by this species reached 4309 μg g^{-1} at 20°C for 6 weeks, and the production at 30°C was low, with a maximum level of 979 μg g^{-1} after 2 weeks (Castellá et al. 1999).

The present study showed the production of MON by *F. temperatum* isolated from maize in Argentina. This species produced the highest level of MON of 5000 μg g^{-1} at 30°C and 0.98 a_w, and the production increased with temperature and incubation time. In agreement with our results, the close related species *F. subglutinans* produced MON at warmer temperatures (25–30°C) growing on maize grains (Kostecki et al. 1999; Schutt 2001). The natural occurrence of MON has been studied more intensively than FUS and BEA because of its higher acute toxicity compared with the other emerging mycotoxins. Levels up to 425 μg g^{-1} have been detected in visually contaminated maize samples (Jestoi 2008). The natural occurrence of MON in various matrices has been reported, detected in maize and maize-derived feeds in levels ranging from 50 to 425 000 μg g^{-1}, in Europe, Africa, China and the United States. The maximum level of MON recorded in maize (530 000 μg kg^{-1}) was detected in *Fusarium*-damaged maize in Poland. More than 300 000 μg kg^{-1} was found in hand-selected, visibly infected kernels from Poland, and up to 25 000 μg kg^{-1} in hand-selected kernels from South Africa (Jestoi 2008). In all these cases, the high

contamination was related to *F. subglutinans*, but before the description of *F. temperatum*. Scudamore et al. (1998) analysed maize gluten and milled maize products, destined for incorporation into animal feed stuffs in the UK, and showed that 60% of samples were contaminated with concentrations up to 4600 μg kg^{-1} of MON. For MON, the results of this study showed that the maximum production by *F. temperatum* is lower than the 50 000 μg kg^{-1} proposed as a maximum level in the diet of broiler chickens (Ledoux et al. 1995).

The existing mainly *in vitro* data on biological activity of FUS, BEA and MON clearly indicate the toxicity of these fungal metabolites. However, there is a lack of *in vivo* toxicity data. In particular, studies on the chronic effects of these mycotoxins are needed to assess their impact on human health. Because of the absence of international limits for emerging mycotoxins in foods, and due to their toxicological effects and the high contamination levels detected, special attention on occurrence is necessary and more research also is needed concerning fungal species and environmental factors responsible for their production in foods.

Data from two-dimensional profiles of a_w by temperature interactions obtained for *F. temperatum* allowed the identification of the areas where climate conditions could indicate a significant risk of mycotoxin accumulation on maize. It appears that when *F. temperatum* grows optimally, but also when it grows under stress conditions, mycotoxin levels could be higher. *F. temperatum* could represent a toxicological risk for maize in the field, during the maize pre-harvest stage and during the ripening stage, when the grains a_w is high (0.98–0.995), and when a_w of the grains is intermediate (0.92–0.97) during the grain drying stage. This species also could represent a toxicological risk during storage if maize is poorly dried and grains have intermediate moisture contents (a_w = 0.92–0.97), because under this condition, colonisation and production of several mycotoxins could occur, reducing the grain quality and safety. Also, it is remarkable the toxicological risk for the possible interaction among the toxins detected.

To ensure future food security and safety, it is crucial to understand how environmental conditions and potential climate change scenarios could affect fungal infection of crops and mycotoxin production by *Fusarium* species. Additionally, in the last years, climate change has been considered as an important factor that can affect food security and safety. The pathways through which climate-related factors may impact on food safety include changes in temperature and precipitation patterns, global temperature warming and precipitation, among others (Tirado et al. 2010). Such changes have an impact on growth and plant susceptibility, and microbial ecology and composition of *Fusarium* species that infect maize kernels, which, in turn, could alter the composition of mycotoxins contaminating infected kernels. Therefore, future efforts toward understanding the ecophysiology and the epidemiology of the infection by *F. temperatum* must focus on more precise relationships between environmental variables and specific components of the disease-cycle in maize. This could allow both short- and long-term planning of mycotoxin control and grain-marketing strategies that ensure safe food and feed supplies.

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