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The effect of fungal competition on colonization of maize grain by *Fusarium moniliforme, F. proliferatum* and *F. graminearum* and on fumonisin B_1 and zearalenone formation

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Abstract

The effect of water activity (0.98, 0.95, 0.93) and temperature (15, 25°C) on fungal growth and toxin production from interactions between isolates of *Fusarium moniliforme* and *F. proliferatum* producing fumonisin, and an isolate of *F. graminearum* producing zearalenone, incubated at the same time on irradiated maize grains were determined in vitro. Populations (CFUs) of *F. moniliforme* and *F. proliferatum* were reduced to a greater or lesser extent by the presence of *F. graminearum* under all conditions tested, while that the presence of *F. moniliforme* or *F. proliferatum* had a minor inhibitory effect on fungal populations of *F. graminearum*. Fumonisin B_1 production by *F. proliferatum* was inhibited under all conditions tested, while fumonisin B_1 production by *F. moniliforme* was inhibited at 15°C and enhanced at 25°C in the presence of *F. graminearum*. The level of zearalenone was not significantly modified in the presence of *F. moniliforme* and *F. proliferatum* under the conditions tested.

Keywords: Fusarium; Fumonisins; Zearalenone; Water activity; Temperature; Maize

1. Introduction

Cereals are among the most suitable substrates for mycotoxin production (Etcheverry et al., 1998). The most frequently isolated *Fusarium* species from maize in temperate climates have been *F. graminearum* and *F. moniliforme,* followed by *F. culmorum, F. subglutinans, F. proliferatum* and *F. equiseti* (Sohn et al., 1999).

F. graminearum produces an estrogrenic mycotoxin, zearalenone (ZEA), as well as trichothecenes. ZEA is usually considered a cereal storage contaminant, and although the toxin can be naturally produced in the field, the majority of toxin production often occurs during cold weather storage of high-moisture feeds carrying the mould. The contaminated grain may have a normal or mouldy appearance (Hollinger and Ekperigin, 1999). On the

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other hand, *F. moniliforme* and *F. proliferatum* are also economically important because they cause maize ear rot and produce fumonisins and other mycotoxins. Fumonisins have been shown to be involved in leukoencephalomalacia in equine species (Thiel et al., 1991, 1992b), associated with pulmonary edema syndrome in swine (Wilson et al., 1990), and implicated in esophageal cancer in humans (Thiel et al., 1992a; Cahagnier et al., 1995).

Fungal growth and mycotoxin production result from the complex interaction of several factors and, therefore, an understanding of each factor involved is essential to understand the overall process and to predict and prevent mycotoxin development (Charmley et al., 1994). Environmental conditions have a major impact on the fungal growth and play a critical role in the mycotoxicosis epidemiology. In addition, mycotoxin production is genetically regulated in response to environmental conditions (Hollinger and Ekperigin, 1999). Temperature and water availability are the primary environmental factors that influence growth and interaction between *Fusarium* spp. and other fungi (Marín et al., 1998a).

Co-occurrence of *Fusarium* mycotoxins has become a significant issue with complex and indeterminate implications for animal and human health. The scientific literature most often reports research on a single-component mycotoxicosis, but clinicians should be aware that the fungi producing these toxins might produce more than one toxin or that multiple toxins might be present in an animal feed. These conditions can result in multiple toxin mycotoxicosis (Hollinger and Ekperigin, 1999).

Marín et al. (1998a) reported that *F. moniliforme* and *F. proliferatum* are dominant over other species which commonly contaminate maize over a wide range of temperature and water availability conditions. Studies on pre-harvest inoculated maize have shown that viable counts and infection of kernels by *F. graminearum* declined while those of *F. moniliforme* increased in mid-season (Miller et al., 1983). Blaney et al. (1986) and Rheeder et al. (1990a,b) also found a negative correlation between the isolation of *F. moniliforme* and *F. graminearum.*

On the other hand, Cuero et al. (1988) suggested that ZEA production by *F. graminearum* in maize, when it was paired in culture with *Aspergillus flavus,* is dependent on temperature, however, the levels of ZEA were not modified in the presence of *A. parasiticus* (Etcheverry et al., 1998).

Little is known about the effects of interactions between *F. moniliforme* and *F. proliferatum* with *F. graminearum* at different water activities (a_w) and temperature on fungal population changes and on fumonisin and ZEA production. The outcome of the interactions between these fungi and toxins production under different environmental conditions may enable a more accurate prediction of which species may initiate spoilage in stored maize.

The aim of this work was to evaluate, under laboratory conditions, the effect of a_w and temperature on fungal growth and toxin production from interactions between isolates of *F. moniliforme* and *F. proliferatum* producing fumonisin, and an isolate of *F. graminearum* producing zearalenone, incubated at the same time on irradiated grains.

2. Materials and methods

2.1. Fungal isolates

Single isolates of three species were used in this study: *F. moniliforme* (25 N) (*F. verticilloides*), *F. proliferatum* (73 N), and *F. graminearum* (CECT 2150, ATCC 26557). All the *Fusarium* species were isolated from maize and *F. moniliforme* and *F. proliferatum* have previously been shown to be high fumonisin producers in culture and maize grain (Marín et al., 1995). All the strains used in the study are maintained in the Food Technology Department fungus collection of the University of Lleida, Spain.

2.2. Grain preparation at different water activity

Spanish dent maize grain was irradiated with 12 kGrays of gamma irradiation and stored aseptically at 4°C. The grain contained no fungal infection or contamination but had retained the ability to germinate. The initial water content of the grain was 13.9% ($a_w = 0.71$).

For all experiments, irradiated maize was weighed into sterile flasks and rehydrated to the desired treatment a_w levels (0.93, 0.95 and 0.98) by addition of sterile distilled water. The amount of water added was calculated from a moisture adsorption curve for

grain. The grain treatments were allowed to equilibrate at 4°C for 48 h, with periodic shaking.

Finally, the a_w values were confirmed by using a water activity meter (AquaLab, Pullman, Washington, USA).

2.3. Inoculation, incubation and growth assessment

Rehydrated maize was placed in sterile Petri plates (20 g per plate, approximately) forming a single layer of grains (Marín et al., 1995). A 5-mm diameter agar disk was taken from the margin of a 5-day-old growing colony of each isolate on malt extract agar and transferred to the centre of each plate. Then, plates containing grain at the same a_w were placed in containers along with beakers containing glycerol–water solutions (Dallyn, 1978) of the same a_w as the plates in order to create an atmosphere with a same equilibrium relative humidity. Containers were incubated at 15 and 25°C. All treatments were repeated three times.

Colonies were measured daily with the aid of a binocular magnifier. Two diameters were obtained from each colony and the growth rates, expressed as mm d^{-1}, were calculated by linear regression of colony radius against time for each strain at each set of conditions tested.

2.4. Inoculation, incubation, and population changes during interaction under different $a_w \times$ temperature conditions

Rehydrated maize was placed in sterile Petri plates (25 g per plate, approximately). For pure culture studies, four agar discs (5-mm diameter) were used, while for mixed cultures, four similar discs were used for each species, distributed on the grain in a fixed pattern. Then, plates containing grain of the same a_w were placed in sealed containers and incubated, as described previously, over 4 weeks at 15 and 25°C. All treatments were repeated twice.

After incubation, plates were taken and analysed for fungal populations (CFU g^{-1}) by dilution plating using MEA (malt extract agar, per 11 distilled water: 20 g of malt extract, 20 g of glucose, 1 g of peptone, pH 5.5) as enumerating media. *Fusarium* were counted on plates bearing a total number of colonies between 5 and 150.

The remaining grains were frozen at −20°C for later fumonisin and ZEA analysis.

2.5. Mycotoxin quantification (Fumonisin B_1 and ZEA)

A modification of the method of Shephard et al. (1990) was followed for Fumonisin B_1 (FB_1) quantification. Subsamples (10 g) were ground and extracted by blending in 20 ml of methanol–water (3 + 1). The extracts were filtered and the filtrate (8 ml) was loaded on a preconditioned strong anion exchange (SAX) column and eluted with 0.5% acetic acid in methanol. The eluate was evaporated to dryness in a rotavapour, redissolved in methanol, and finally evaporated under a gentle stream of nitrogen and dissolved in methanol for HPLC. The sample was coupled to OPA (phthaldialdehyde) reagent and assayed by HPLC by comparison with external standards using methanol–0.1 M dihydrogen sodium phosphate (3 + 1) (pH 3.35) as the mobile phase at a 0.7 ml min^{-1} flow-rate. The reference standard of FB_1 was purchased from Sigma (St. Louis, MO, USA).

For ZEA quantification, the AOAC method for α-zearalenol and zearalenone in maize was followed (AOAC, 1997).

2.6. Dry matter determination

The percentage of dry matter from each sample was determined by drying subsamples of approximately 10 g at 105°C for 17 h (ISTA, 1976). Thus, all results are presented on a dry weight basis.

2.7. Statistical analyses of the data

Analysis of variance were made for colony radii after 4 days, CFUs g^{-1}, zearalenone, and fumonisin concentrations by using SAS program version 6.12 (SAS Institute, Inc., Cary, NC, USA). CFU data were transformed prior to analysis by $y = \log(\text{CFU } g^{-1})$. Finally, correlation analyses were carried out between CFU and mycotoxin production in pure cultures. For mixed cultures, analysis of correlation was carried out between the amount of mycotoxins presents and the percentage of inhibition of fungal populations.

3. Results

3.1. Growth rates of Fusarium spp.

Generally, growth rates increased from 0.93 to 0.98 a_w at both temperatures on irradiated maize grain. Furthermore, the three *Fusarium* species examined grew faster at 25°C than at 15°C (Table 1). The highest growth rates obtained for *F. moniliforme, F. proliferatum,* and *F. graminearum* were 4.1, 4.0, and 9.8 mm day^{-1}, respectively, at 0.98 a_w and 25°C. *F. graminearum* grew faster than *F. moni1iforme* and *F. proliferatum* isolates under all the conditions tested.

3.2. Effect of the presence of F. graminearum on populations and FB_1 production by F. moniliforme

All single factors (a_w, temperature and presence of *F. graminearum*) had a significant effect (Table 2) on fungal populations (CFU per gram of grain) of *F. moniliforme.* There were significantly more CFUs of *F. moniliforme* at 25°C than at 15°C ($P < 0.01$). At 15°C more CFUs of *F. moniliforme* were present at 0.98 than at 0.95 or 0.93 a_w, whereas at 25°C populations at different a_w were not significantly different.

The presence of *F. graminearum* decreased the number of CFUs per gram of grain of *F. moniliforme* under all the conditions tested (7% mean reduction) (Fig. 1), but no significative differences were found in the percentages of reduction at different temperatures or a_w.

For the formation of fumomisin B_1 (FB_1) only a_w and the presence of *F. graminearum* at different temperature levels ($T \times I$) were significant (Table 2). FB_1 production increased at increasing a_w values. At 15°C, FB_1 production by *F. moniliforme* decreased (72% mean reduction), due to the presence of *F. graminearum.* However, at 25°C FB_1, production was significantly enhanced (150% mean increase), mainly at 0.95 and 0.98 a_w (Fig. 1).

Table 2
Analysis of variance of effect of water activity (a_w), temperature (t), and presence of *Fusarium graminearum* (I) on population (CFUs) and fumonisin B_1 production by *F. moniliforme*[a]

Source of variation	*df*	CFUs		FB_1 production	
		MS	F	MS	F
t	1	8.45	241.11**	21.71	0.92
a_w	2	1.35	38.38**	351.41	14.85**
$t \times a_w$	2	0.43	12.35**	94.01	3.97
I	1	1.77	50.41**	0.52	0.02
$t \times I$	1	0.004	0.13	667.82	28.21**
$a_w \times I$	2	0	0.01	74.8	3.16
$t \times a_w \times I$	2	0.27	7.67**	74.5	3.15

[a] MS = Square. *$P < 0.05$, **$P < 0.01$.

3.2.1. Effect of the presence of F. graminearum on populations and FB_1 production by F. proliferatum

Concerning CFUs, all single factors (a_w, temperature, and presence of *F. graminearum)* as well as two-way interactions were significant (Table 3). More CFUs of *F. proliferatum* were present at 25°C than at 15°C and more at 0.98 than 0.95 or 0.93 a_w. In the presence of *F. graminearum,* the number of CFUs decreased almost under all conditions tested, but it was more evident at 15°C than at 25°C (11 and 5% log CFU reduction, respectively) (Fig. 2).

All single factors, as well as their two- and three-way interactions, had a significant effect on FB_1 production (Table 3). In general, more FB_1, was produced at 15°C than at 25°C and when a_w in-

Table 1
Effect of water activity and temperature on growth rates of three *Fusarium* species (mm day^{-1})

Temp. (°C)	a_w	*F. moniliforme*	*F. proliferatum*	*F. graminearum*
15	0.93	0.15	0.27	0.70
	0.95	0.76	0.85	2.48
	0.98	2.22	2.27	5.49
25	0.93	0.67	0.85	1.50
	0.95	2.52	2.32	5.82
	0.98	4.12	4.03	9.79

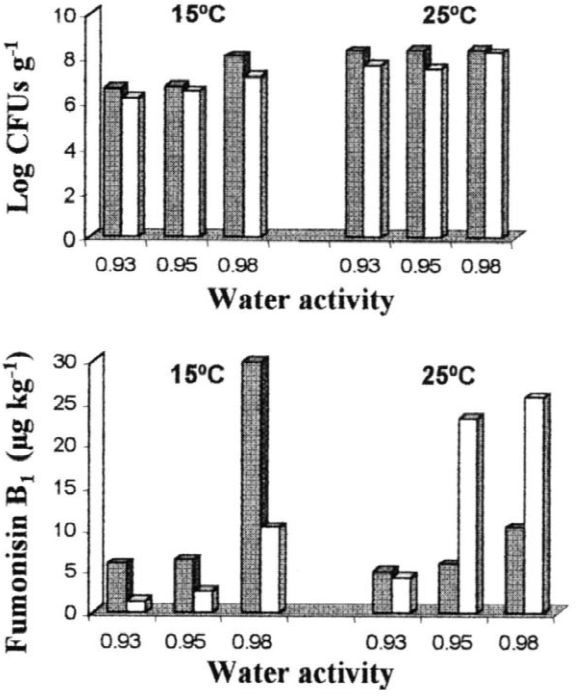

Fig. 1. Effect of coinoculation of *Fusarium graminearum*, water activity and temperature on growth and fumonisin B_1 production by *F. moniliforme* on irradiated maize grain incubated for 28 days. Single inoculum (▨); mixed inoculum (□).

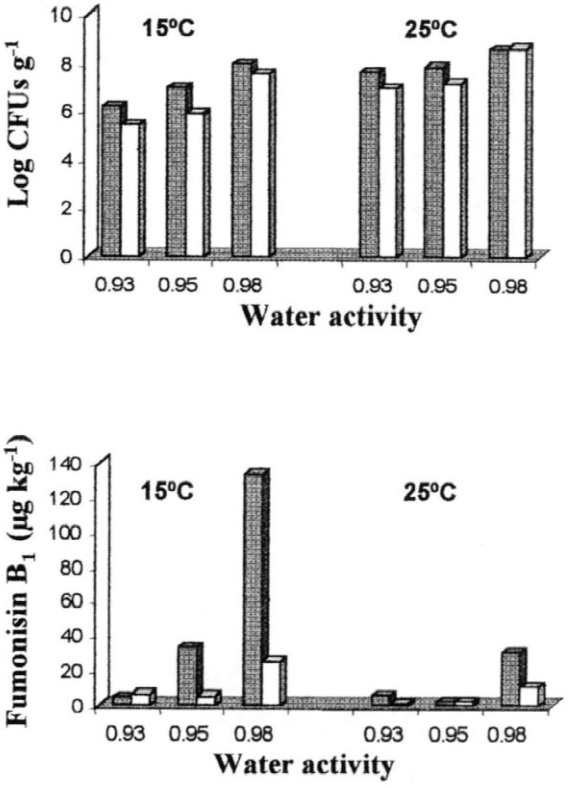

Fig. 2. Effect of coinoculation of *Fusarium graminearum*, water activity and temperature on growth and FB_1, production by *F. proliferatum* on irradiated maize grain incubated for 28 days. Single inoculum (▨); mixed inoculum (□).

Table 3
Analysis of variance of effect of water activity (a_w), temperature (t), and presence of *Fusarium graminearum* (I) on population (CFUs) and fumonisin B_1 production by *F. proliferatum*[a]

Source of variation	*df*	CFUs		FB_1 production	
		MS	F	MS	F
t	1	7.46	424.24**	4016.14	31.64**
a_w	2	5.74	327.12**	4842.08	38.15**
$t \times a_w$	2	0.24	13.62**	1735.16	13.67**
I	1	1.93	109.82**	4246.35	33.45**
$t \times I$	1	0.18	10.08**	1958.55	15.43**
$a_w \times I$	2	0.23	13.12**	21.72	17.11**
$t \times a_w \times I$	2	0.02	1.31	1120.22	8.83**

[a] MS = Square. $*P < 0.05$, $**P < 0.01$.

creased. In the presence of *F. graminearum*, FB_1 production decreased under almost all conditions, at 15°C the inhibition was higher than at 25°C (78 and 65% mean reduction, respectively), and was more important at the highest a_w (Fig. 2).

3.2.1.1. Effect of the presence of F. moniliforme or F. proliferatum on populations and ZEA production by F. graminearum

Concerning CFUs, all single factors (a_w, temperature and presence of *F. moniliforme* or *F. proliferatum*) as well as their two- and three-way interactions were significant (Table 4), except for temperature $\times a_w$. In pure culture, more CFUs of *F. graminearum* were present on maize at 0.98 a_w rather than at 0.95 and 0.93 and at 25°C than at 15°C.

In the presence of *F. moniliforme*, CFUs of *F. graminearum* were reduced significantly at 15 and 25°C, while the presence of *F. proliferatum* only had a significant inhibitory effect at 25°C.

The effect of a_w and temperature, as well as their two-way interaction were significant (Table 4), but in the presence of *F. moniliforme* or *F. proliferatum,*

Table 4
Analysis of variance of effect of water activity (a_w), temperature (t), and presence of *Fusarium moniliforme* or *F. proliferatum* (I) on population (CFUs) and zearalenone production by *F. graminearum*[a]

Source of variation	*df*	CFUs		Zearalenone production	
		MS	F	MS	F
t	1	4.61	443.03**	1626.26	5.5*
a_w	2	1.33	127.8**	15781.11	53.32**
$t \times a_w$	2	0.03	3.16	3809.01	12.87**
I	2	0.1	9.24**	468.23	1.58
$t \times I$	2	0.05	4.33*	333.67	1.13
$a_w \times I$	4	0.13	11.97**	336.55	1.14
$t \times a_w \times I$	4	0.1	9.l**	386.26	1.31

[a] MS = Square. $*P < 0.05$, $**P < 0.01$.

ZEA production by *F. graminearum* was not significantly affected (Table 4). However, ZEA production, in the presence of *F. moniliforme* or *F. proliferatum* was under certain conditions higher than in the pure culture. There was more ZEA production by *F. graminearum* at 15°C than at 25°C and more at 0.98 than at 0.95 and 0.93 a_w (Fig. 3).

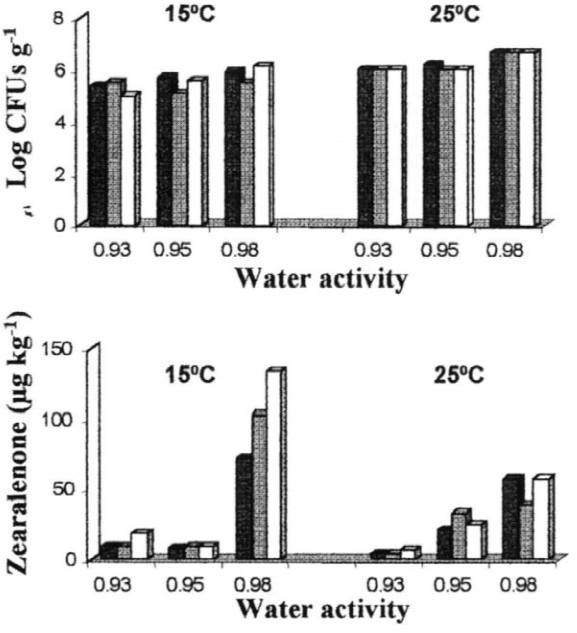

Fig. 3. Effect of coinoculation of *Fusariurm moniliforme* and *F. proliferatum* isolates, water activity and temperature on growth and zearalenone production by *F. graminearum* on irradiated maize grain incubated for 28 days. Single inoculum (■); mixed inoculum with *F. moniliforme* (▨); mixed inoculum with *F. proliferatum* (□).

3.2.1.2. Correlation analyses

In the pure culture no correlation was seen between FB_1, production and fungal populations of *F. moniliforme* and *F. proliferatum*, nor between ZEA production and CFUs of *F. graminearum*. The percentage of reduction of CFUs of *F. moniliforme*, *F. proliferatum*, and *F. graminearum* in mixed cultures were not correlated significantly with ZEA or FB_1 concentration.

4. Discussion

Our data showed that *F. moniliforme* and *F. proliferatum* populations were reduced to a greater or lesser extent by the presence of *F. graminearum* under all conditions tested, while the presence of *F. moniliforme* or *F. proliferatum* had a limited inhibitory effect on fungal populations of *F. graminearum*.

It has been suggested that the significant correlation between the presence of different *Fusarium* species over locations may be due not to interactions, but rather as the influence of environmental conditions (Rheeder et al., 1990a). Previous reports from South Africa (Marasas et al., 1979; Rheeder et al., 1990b) indicated that *F. moniliforme* was more adapted to a warm and dry climate than *F. graminearum*. Our data also reflected that development and toxin production by the different *Fusarium* species were differently affected by environmental conditions. However, besides abiotic factors, such as a_w and temperature, it was found that fungal interaction is a biotic factor that significantly affected fungal development in grain.

Although growth rate and populations are probably useful measures to assess fungal colonization by *Fusarium* spp. in maize grain, they do not correlate well with fungal colonization in mixed cultures. *F. graminearum* had a higher growth rate than *F. moniliforme* or *F. proliferatum* under all conditions tested, while *F. moniliforme* or *F. proliferatum* sporulated more than *F. graminearum*. The ability to grow fast and to sporulate abundantly are both important characteristics for occupation of an ecological niche (Magan and Lacey, 1984; Ramakrishna et al., 1993).

Marín et al. (1998a) suggested that *F. graminearum* at 15°C might have a competitive

advantage over *F. moniliforme* and *F. proliferatum* probably due to the ability to utilise resources that these latter species cannot. By contrast, at 25–30°C, these *Fusarium* species appeared to coexist in the same niche. The outcome of parings of *F. moniliforme* and *F. proliferatum* with *F. graminearum* resulted in mutually antagonistic interactions upon contact (Marín et al., 1998a). It has been suggested that *F. moniliforme* protects maize seedlings against infection by *F. graminearum* (Van Wyk et al., 1988).

Our data showed no good correlation between CFUs and mycotoxin production in the pure culture of either of the species studied, meaning that in this case there is no correlation between sporulation and toxin formation.

On the other hand, the absence of a correlation between the percentage of reduction of *F. moniliforme, F. proliferatum*, and *F. graminearum* populations in mixed culture and ZEA and FB_1 concentrations, respectively, suggests that neither FB_1 nor ZEA might act as fungal inhibitors, although their production is affected by fungal interactions under certain environmental conditions. Similar results were obtained by Marín et al. (1998b) working with fumonisin-producers and a range of *Aspergillus* and *Penicillium* species.

There are many conflicting reports about the effects of fungal interactions on mycotoxin production. We have shown for first time the effect of coinoculation of different *Fusarium* species on FB_1 and ZEA production. Our results revealed different behaviour in FB_1 production by *F. moniliforme* and *F. proliferatum* in the presence of *F. graminearum*. FB_1 production by *F. proliferatum* was inhibited under all the conditions tested. On the other hand, *F. graminearum* was able to inhibit the FB_1 production by *F. moniliforme* at 15°C, while at 25°C it was significantly increased. Marín et al. (1998b) also found a significant increase in fumonisin concentration when fungal interaction occurred, mainly with some *Aspergillus* species and at high water availability.

In our study, the level of ZEA was not significantly modified in the presence of *F. moniliforme* and *F. proliferatum* at any of the conditions tested. Etcheverry et al. (1998) also found that ZEA production was not affected when *F. graminearum* was paired with *A. parasiticus* at 28°C and 0.97 a_w. Conversely, ZEA production by *F. graminearum* was markedly decreased in a paired culture with *A. flavus* at 16°C but not at 25°C (Cuero et al., 1988). Our results suggest that when the environmental conditions in maize grain are favourable and the potentially toxin producers strains *F. proliferatum* and *F. graminearum* are found together a decrease in the concentration of FB_1 may happen, but no change on the level of ZEA will happen. Nevertheless, if *F. moniliforme* and *F. graminearum* are found together, the production of FB_1 under certain conditions might be enhanced, while the concentration of ZEA might remain unchanged.

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