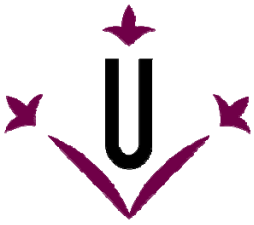

The effect of enhanced carotenoid content of transgenic maize grain on fungal colonization and mycotoxin content

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Abstract

Novel strategies that address vitamin A deficiency have been developed, such as high-carotenoid maize, a biofortified transgenic maize line rich in carotenoids generated by genetic transformation. The South African white maize inbred (M37W), which is devoid of carotenoids, was engineered to accumulate high levels of β-carotene (provitamin A), lutein and zeaxanthin. Maize seeds contaminated with fumonisins and other mycotoxins pose a serious threat to both humans and livestock. During three consecutive harvests, the fungal incidence and the fumonisin and aflatoxin content of maize seeds grown in an experimental field in Catalonia (northeastern Spain) were evaluated. Fungal infection was similar in high-carotenoid maize and its isogenic line, with *Fusarium verticillioides* being the most prevalent fungus in all the harvests. Neither *Aspergillus* spp. nor aflatoxin contamination was found. Fumonisin levels were lower in high-carotenoid than in its isogenic line, but this reduction was statistically significant in only two of the three years of study. Our results suggest that high carotenoid content reduces fumonisin levels in maize grains.

Keywords

Maize, carotenoids, fumonisins, aflatoxins, *Fusarium.*

Abbreviations

AFs, aflatoxins; FBs, fumonisins; FB_1, fumonisin B_1; FB_2, fumonisin B_2; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; HPLC, high-performance liquid chromatography.

Introduction

In many developing countries, populations subsist on a cereal-dominated diet with low levels of nutrients, including β-carotene. Chronically insufficient vitamin A intake in the diet can lead to disorders such as xerophthalmia, anemia and weakened resistance to infection. It has been estimated that vitamin A deficiency (VAD) affects up to one third of the world's pre-school-age children and up to 15% of pregnant women. VAD is prevalent worldwide but is particularly severe in Africa and South-East Asia (WHO, 2009).

In sub-Saharan Africa, white maize is the predominant food maize used, while yellow maize, with a higher carotenoid content (but with no provitamin A carotenoids), is mainly used as animal feed (Naqvi et al. 2009). The South African white endosperm maize inbred M37W, which lacks carotenoids in the endosperm because of the absence of the enzyme phytoene synthase (necessary for the biosynthesis of these metabolites), was used as a basis to create the high-carotenoid maize through combinatorial nuclear transformation (as described in Zhu et al. 2008). *Zmpsy1* (*Zea mays* phytoene synthase 1) and *PacrtI* (*Pantoea ananatis* phytoene desaturase) controlled by endosperm-specific promoters were introduced into M37W inbred to generate the high-carotenoid maize. The maize line used in the current experiments is able to provide the recommended daily intake of provitamin A in 200 g of grain (Naqvi et al. 2009).

Mycotoxins occurring in food commodities are low-molecular-weight natural products synthesized as secondary metabolites by filamentous fungi. The most important toxins are produced by species in the genera *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria*, which can grow on a variety of crops, including cereals, and can enter the food chain during preharvest, harvest, drying, or storage, and even in manufactured products, thus offering many opportunities to contaminate food and feed (Shephard 2008). Maize (*Zea mays* L.) seeds are often contaminated with fumonisins (FBs), produced primarily by *Fusarium verticillioides* and *F. proliferatum*. However, other mycotoxins may frequently be present alone or in combination with FBs, including aflatoxins (AFs), deoxynivalenol, zearalenone, and some recently discovered *Fusarium* metabolites collectively known as emergent mycotoxins, such as moniliformin, beauvericin, enniatins and fusaproliferin. AFs are mainly produced by *Aspergillus flavus*, which synthetizes type B aflatoxins as well as cyclopiazonic acid, depending on the strain, and by *A. parasiticus*, which synthetizes both type B and type G aflatoxins but not cyclopiazonic acid (Desjardins 2006; Marin et al. 2013).

Fumonisin-contaminated maize is toxic, causing leukoencephalomalacia in equines (Kellerman et al. 1990), pulmonary edema in swine (Harrison et al. 1990) and cancer in humans, especially in areas where maize is the staple diet (Marasas 2001). The mode of action of FBs is directly or indirectly linked to the effects of FBs on the sphingolipid metabolism (Wang et al. 1991). Fumonisin B_1 (FB_1) is potentially carcinogenic in humans (Group 2B), according to the International Agency for Research on Cancer (IARC) (IARC 2002). It is also the most prevalent *Fusarium* toxin in maize. Human exposure to FBs in maize is common worldwide. In 2001, several countries submitted information on the concentration of FBs in maize and maize-derived foods, and FBs were detected in more than 60% of all food products (JECFA 2001).

AFs are hepatotoxic, carcinogenic, immunosuppressive and anti-nutritional contaminants found in some staple foods. Chronic exposure to AFs can compromise the immunity and nutritional status of farm and laboratory animals, reducing growth and productivity. AFs are potent carcinogens in all animal species studied thus far (mice, rats, hamsters, fish, piglets and chickens) (Williams et al. 2004; Marin et al. 2013). According to the IARC (IARC 2012), there is sufficient evidence to classify the main AFs (AFB_1, AFB_2, AFG_1 and AFG_2) as carcinogenic in humans (Group 1). Aflatoxin B_1 (AFB_1) is considered the most potent aflatoxin and is also the most carcinogenic natural compound known.

Maize is the most important source of fumonisin contamination in foods, and infected kernels pose a serious threat to both humans and livestock, leading to the importance of evaluating the susceptibility to contamination of new maize varieties. It is unknown the vulnerability to fungal infection and mycotoxin contamination of high-carotenoid maize. Thus, this work aims to evaluate, for three consecutive harvest seasons, the fungal incidence and the fumonisin and aflatoxin presence in high-carotenoid maize and its isogenic control line in open field experiments.

Material and methods

Maize

The South African white maize inbred M37W (control maize), which is essentially devoid of carotenoids, and its engineered derivative (high-carotenoid maize), which accumulates high levels of β-carotene, lutein and zeaxanthin (Zhu et al. 2008), were cultivated in an experimental field trial in Lleida (Catalonia, northeastern Spain) during three consecutive years (2013, 2014 and 2015). Maize seeds were sown in

May (2013 and 2014) or June (2015) and maize plants harvested in November in all the years of the study. The experimental field had a Latin square design with four replicates accommodating a two-way factorial. There were six-row plots and 28 plants per row, being the surface area of each plot 7×5 m^2. Every plot was randomly sampled at harvest to obtain a representative sample, and the samples from each plot were combined to obtain an aggregate sample of each type of maize for the mycobiota analysis. After harvest, maize cobs were dried at low temperature (35 ºC) for 24 hours in a drying chamber, sampled according to the Commission Regulation No. 401/2006 (European Commission 2006a), milled (Ras® Mill, Romer® Labs Inc., MO, USA) and frozen at -18 °C until analysis by high-performance liquid chromatography (HPLC).

Moisture and water activity determinations

The moisture content of the field samples was determined by oven drying according to the Association of Official Analytical Chemists method (AOAC International 2005). The water activity (a_w) was determined using an Aqualab CX2T (Decagon Devices, Pullman, WA, USA).

Meteorological data

Meteorological data were obtained from the nearest meteorological station to the experimental field through the weather network of Catalonia (Servei Metereològic de Catalunya). Maximum, mean and minimum temperatures and rainfall from May to November were recorded to describe the environmental conditions during the trials (Fig. 1).

Fig. 1 Meteorological data (temperature and rainfall) from May to November in the experimental field in Lleida (Catalonia, Spain) in 2013, 2014 and 2015.

Mycobiota determination

One hundred grains from each of the two maize varieties were randomly chosen immediately after harvest and decontaminated by immersion in 3% sodium hypochlorite (NaClO) (GPR Rectapur®, VWR Chemicals, Barcelona, Spain) followed by two rinses in sterile water. Kernels were plated in groups of five in dichloran rose-bengal chloramphenicol agar media (DRBC) Petri plates, which were then incubated for 7 days at 25 ºC in darkness. Colonies of developing fungi were isolated in potato dextrose agar (PDA), and the plates were incubated for 7 days at 25 ºC in darkness. Classification into genera was

performed according to Pitt and Hocking (2009). The species level was identified using molecular biology techniques. The incidence of fungal infection was calculated as percentage considering that one hundred grains from each of the two maize varieties were isolated each harvest.

DNA extraction, amplification and identification

Pure cultures were grown in 500 µL malt extract broth in Eppendorf tubes (2% w/v malt extract, 1% w/v peptone, 2% w/v glucose) for 2 days at 25 °C in darkness. The mycelial extract was recovered by centrifugation (14000 rpm, 10 min), and 300 µL of TNES buffer [200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.5% w/v sodium dodecyl sulfate (SDS)] was added. The mycelial suspension was lysed by vortexing with five 2.8 mm Precellys metal beads for 10 min. After centrifugation (14000 rpm, 10 min), 150 µL of 3 mM sodium acetate (pH 5.2) was added to the supernatant. The supernatant was stored at -20 °C for 10 min and then centrifuged (14000 rpm, 10 min). The DNA-containing supernatant was transferred to a new tube, and nucleic acids were precipitated by the addition of 1 volume of isopropanol. After a 5 min incubation at room temperature, the DNA suspension was centrifuged (14000 rpm, 10 min). The DNA pellet was washed with 70% ethanol. The resulting pellet was air-dried, and the DNA was resuspended in 50 µL TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). After extraction, the DNA concentrations were determined using a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Firstly, all the isolates were subjected to amplification reactions for *Fusarium* identification. Secondly, amplification reactions for DNA sequencing were carried out with the isolates whose amplification product was negative for the specific *Fusarium* primers tested. Amplification reactions for *Fusarium* identification were conducted in volumes of 10 µL containing 10 ng template DNA, 0.5 µL of each primer (10 µM), 1 µl of 10x PCR buffer, 0.25 µL dNTPs (10 mM) and 0.1 µL Taq DNA polymerase (5 U μL^{-1}) supplied by the manufacturer (Bioron GmbH, Ludwigshafen, Germany). The PCR buffer had the following composition 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8.8, 0.1 % Tween-20, 25 mM $MgCl_2$. To identify *Fusarium* isolates, specific primers were used (Table 1). Amplification reactions for DNA sequencing were conducted in volumes of 100 µL containing 10 ng template DNA, 4 µL of each primer (10 µM), 10 µL of 10x PCR buffer, 2 µL dNTPs (10 mM) and 0.75 µL Taq DNA polymerase (5 U μL^{-1}). The primers used for DNA sequencing are summarized in Table 2.

All PCR assays were performed using a GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA, USA) under the conditions summarized in Table 3. Amplification products were detected by electrophoresis on 1.5% agarose ethidium- bromide gels in TAE 1x Buffer (40 mM Tris-acetate and 1 mM EDTA). A TrackIt™ 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used as molecular size marker. For DNA sequencing, PCR products were cleaned with the GeneJet PCR purification kit (Thermo Scientific, Waltham, MA, USA). The purified PCR products were sequenced by the company Macrogen Europe (Amsterdam, Netherlands). Sequences were analyzed and aligned using the Blast® search tool (National Center for Biotechnology Information, Bethesda, MD, USA).

Extraction, detection and quantification of FBs and AFs by HPLC

For fumonisin analysis, each sample (10 g maize + 1 g of sodium chloride) was extracted with 50 mL of acetonitrile: methanol: water (25: 25: 50 v/v/v) by blending for 20 minutes. The extracts were filtered, and 10 mL of each filtrate was diluted with 40 mL of phosphate buffered saline (PBS) solution at pH 7.4. For aflatoxin analysis, each sample (5 g maize) was extracted with 15 mL of methanol: water (60: 40 v/v) by blending for 10 minutes. The extracts were filtered, and 2 mL of each filtrate was diluted with 14 mL of PBS solution. In both cases, diluted extracts were cleaned up using immunoaffinity columns, Fumoniprep® for FBs and Easi-Extract® aflatoxin for AFs (R-Biopharm AG, Darmstadt, Germany). The filtrates were passed through the column at a flow rate of 2 mL/min, and the columns were then washed by passing 20 mL of PBS through at a flow rate of 5 mL/min. Finally, the toxins were eluted using 3 mL of methanol slowly passed through the column, and the solvent was evaporated to dryness under a nitrogen stream. The dried samples were re-dissolved in 500 µL methanol: water (50: 50 v/v), and a volume of 200 µL was injected into the HPLC system.

FBs (FB_1 + FB_2) and AFs (AFB_1 + AFB_2 + AFG_1 + AFG_2) were detected and quantified separately using an HPLC system (Waters, Milford, MA, USA) and a C18 column (5 µm Waters Spherisorb, 4.6 x 250 mm ODS2). For fluorescence detection, a Waters 2475 module was used at the following wavelengths: ʎexc 362 nm and ʎem 440 nm for AFs; ʎexc 335 nm and ʎem 440 nm for FBs. FBs were manually derivatized with ortho-phthalaldehyde (OPA) (Sydenham et al. 1996), while AFs were photochemically derivatized (UVE™, LC Tech GmbH, Dorfen, Germany). The analysis was performed under isocratic conditions at a flow rate of 1 mL/min, and the mobile phase used was water: acetonitrile: methanol (70: 17: 17) for AFs and methanol: 0.1 M sodium dihydrogen phosphate solution (77: 23) (adjusted to pH 3.35

with orthophosphoric acid) for FBs. The detection limits were established based on a signal-to-noise ratio of 3: 1 and were 0.25 ng/g for AFB_1 and AFG_1, 0.15 ng/g for AFB_2 and AFG_2, 0.02 µg/g for FB_1 and 0.04 µg/g for FB_2. Quantification was performed using a software integrator (Empower, Milford, MA, USA). The analytical methods were validated according to the Commission Regulation No. 401/2006 (European Commission 2006a). The recovery percentage obtained was considered to express the results.

Statistical analysis.

ANOVA statistical tests and t-test for means comparison were employed (JMP® Pro 12 SAS institute, 2015). Differences among means with $p < 0.05$ were accepted as representing statistically significant differences.

Results

Moisture and water activity

The water activity and moisture values of the field samples were similar for both types of maize, high-carotenoid and its isogenic line (Table 4). However, the moisture content was higher during the third year of study in both types of maize, probably due to the delayed sowing time.

Mycobiota incidence and identification

The incidence of fungal infection was the same for both types of maize in 2013 (90%). In 2014, it was 86 and 81% for control and high-carotenoid maize, respectively. In 2015, it was 100 and 86% for control and high-carotenoid maize, respectively. Thus, the percentage of infected grains was 5% and 14% higher in control maize than in high-carotenoid maize in 2014 and 2015, respectively.

Fusarium was the dominant genus isolated, present in more than 60% of both types of maize in all years of the study. The most common species isolated within this genus was *F. verticillioides*, although there was a decrease in its presence in favor of *F. proliferatum* in control maize in 2014. Other *Fusarium* spp., such as *F. subglutinans* and *F. poae*, were occasionally isolated. Other less frequently isolated fungi were *Acremonium strictum*, *Chaetomium globosum*, *Epicocum nigrum* and *Lecanicillium muscarium*, and other occasionally isolated genera were *Cladosporium*, *Penicillium, Alternaria*, *Talaromyces*, *Galactomyces* and *Sarocladium.* Nonetheless, all of these fungi accounted for less than 15% of total infection in both types of maize in all the years of study, except for high-carotenoid maize in 2013, in which the presence

of other fungi increased to 30%, *Acremonium strictum* the fungus responsible for half of this contamination. There was no *Aspergillus* spp. presence in any of the years studied. Fig. 2 shows the identified mycobiota in both types of maize during the trials.

Fig. 2 Mycobiota identification in control and high-carotenoid maize during the trials.

Fumonisin and aflatoxin contamination

Both types of maize were contaminated with FBs in all three years of the study, but control maize was 1.4-, 2.4- and 2-fold more contaminated than high-carotenoid maize in 2013, 2014 and 2015, respectively. This difference was statistically significant ($p < 0.05$) in 2014 and 2015 (Fig. 3). On the other hand, there was no contamination by AFs in any of the years.

Fig. 3 Fumonisin levels ($FB_1 + FB_2$, in mg/kg) in control and high-carotenoid maize during the trials. The values shown are the mean and standard error for each treatment (n=3). The asterisk indicates statistically significant differences between types of maize ($p < 0.05$).

Discussion

High-carotenoid maize can be a sustainable and cost-effective alternative to vitamin A supplementation in developing countries, as it can provide the recommended daily intake of provitamin A in 200 g of grain (Naqvi et al. 2009). Its vulnerability to fungal infection and mycotoxin contamination compared to its isogenic line has been evaluated in open field trials.

This study has shown slight differences in fungal infection between types of maize, with *F. verticillioides* being the most common species isolated in all three harvest seasons. Our results are consistent with previous studies that established that *F. verticillioides* and *F. proliferatum* are the most prevalent fungi in maize in our area (Marín et al. 2012), although Bakan et al. (2002) found that *F. proliferatum* was more common than *F. verticillioides* in other fields located in northeastern Spain. Fumonisin contamination was detected in all three years of the study, agreeing with previous studies that reported fumonisin contamination in maize products in the Spanish market (Velluti et al. 2001; Cano-Sancho et al. 2012). Considering the results obtained in our experiments, only the control maize harvested in 2014 had fumonisin levels above the maximum allowed level established by the European legislation for unprocessed maize (4000 µg/kg) (European Commission 2006b, 2007). In 2014, there was higher

fumonisin contamination in both types of maize compared to the other harvest seasons that could be explained by the environmental conditions, as high rainfall was recorded two months before harvest in 2014, whereas the climatic conditions in 2013 and 2015 were quite similar (Fig. 1). This observation is in accordance with Cao et al. (2014) which pointed out that fumonisin accumulation is favored by hard rainfall during kernel drying and Marín et al. (2004) which reviewed the ecophysiology of fumonisin-producing *Fusarium* species and reported that during the ripening and final drying in the field, there are many opportunities for FB_1 production.

The differences in fumonisin contamination between types of maize were significant in two of the three years of the study and could be related to their differences in carotenoid content. Both types of maize had the same genetic background, as control maize was the basis for creating the high-carotenoid maize. Thus, the only difference between them was the carotenoid content which was 88 ± 8 µg/g dw (dry weight) for high-carotenoid maize and 1± 0.5 µg/g dw for control maize. Zeaxanthin levels were 23 and 0.3 µg/g dw, and lutein levels were 9 and 0.6 µg/g dw for high-carotenoid and control maize, respectively. Provitamin-A carotenoids were only detected in high-carotenoid maize (5.9 ± 2 and 3.7 ± 0.6 µg/g dw for β-carotene and β-cryptoxanthin, respectively) (Zanga et al. personal communication).

Carotenoids are well-known antioxidants (Stahl and Sies 2003), although the effect of carotenoids on mycotoxin biosynthesis has been scarcely studied. Fungi are often exposed to unfavorable environmental conditions, such as oxidative stress. It has been suggested that oxidative stress may be related to mycotoxin production, some toxins may be produced to counteract the excessive accumulation of reactive oxygen species (Reverberi et al. 2010). Thus, as fumonisin biosynthesis can be stimulated by oxidative stress, the presence of antioxidants might therefore help to disrupt toxin accumulation (Picot et al. 2010). This is supported by a recent review which has highlighted that antioxidant compounds such as carotenoids can quench oxygen free radicals produced by plant cells as a defense response, contributing to reduce oxidative stress that modulates toxin biosynthesis. Moreover, these lipophilic secondary metabolites can scavenge lipid peroxyl free radicals, reducing the lipid peroxidation (Atasanova-Penichon et al. 2016).

Regarding FBs, Picot et al. (2013) studied the potential involvement of maize antioxidants (ferulic acid, tocopherols and carotenoids) in resistance to *Fusarium* ear rot and fumonisin accumulation. Lutein (0.4 µg/mL), zeaxanthin (0.4 µg/mL) and β-carotene (0.2 µg/mL) activity levels were evaluated *in vitro*. In

lutein-supplemented liquid cultures, a reduction (52%) was observed in fumonisin production by one of the three strains of *F. verticillioides* tested, but the difference was not significant compared to the control treatments. Delgado et al. (2014) found a statistically significant positive correlation between lutein content and *Fusarium* mycotoxins in durum wheat, but FBs were not evaluated. With respect to AFs, it has been reported that the presence of carotenoids may reduce aflatoxin biosynthesis by *A. flavus* (Norton 1997; Wicklow et al. 1998).

Many factors can modulate fumonisin biosynthesis: genes such as the FUM gene cluster, ecophysiological factors such as water activity and temperature, physicochemical and nutritional factors such as pH and C:N ratio, and plant defense metabolites (Picot et al. 2010). Endogenous plant compounds, both constitutive and induced in response to pathogen infection, may disrupt toxin biosynthesis (Boutigny et al. 2008). It has been reported that the physiological stages of the maize kernel play a role in the regulation of fumonisin biosynthesis. Fungal infection can occur from the blister stage, but fumonisin production is not initiated until the dough stage. It has been shown that the dent stage can increase fumonisin biosynthesis (Picot et al. 2011). Considering these previous results, the presence of carotenoids has been demonstrated in maize kernels from the blister to the final mature stages. Therefore, carotenoids are expected to be present when fumonisin biosynthesis occurs (Picot et al. 2013).

The carotenoid enhancement in transgenic lines has been shown to affect starch metabolism. In transgenic lines with a total carotenoid content similar to the high-carotenoid maize, the total starch content was found to decrease by up to 8% compared to the isogenic maize (Berman 2016). Many hypotheses have been formulated regarding the role of the endosperm composition in fumonisin biosynthesis (Santiago et al. 2015). Bluhm and Woloshuk (2005) noted that amylopectin presence might induce fumonisin production by *F. verticillioides*. Blandino and Reyneri (2007) studied *waxy* maize, which produces an endosperm granule that contains almost 100% amylopectin, showing that the waxy hybrids were more susceptible to fumonisin accumulation than the near-isogenic lines. However, Picot et al. (2011) suggested that under field conditions, amylopectin is not the only component that favors fumonisin biosynthesis. The increase in fumonisin production appeared three weeks after the completion of the kernel with amylopectin, suggesting that other mechanisms such as pH may be involved. Carotenoid-enriched maize lines had a slight difference in starch content compared to their isogenic line. It would be interesting to study in detail whether starch content and composition in carotenoid-enriched maize lines could play a role in fumonisin reduction.

Carotenoids, as potent maize antioxidant compounds, may disrupt fumonisin biosynthesis or accumulation in maize plants. It has been demonstrated that the high-carotenoid maize not only does not differ from its isogenic line in fungal infection but also has a positive effect in terms of fumonisin reduction. Nonetheless, it is important to bear in mind that carotenoids may play a role together or individually. Further studies are necessary to clarify the effect of carotenoids on fumonisin production.

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Table 1 Specific primers for *Fusarium* identification.

Target	Primer name	Sequence (5'−3')	Size (bp)	Source
F. verticillioides	VERT-1	GTCAGAATCCATGCCAGAACG	800	Patiño et al. 2004
	VERT-2	CACCCGCAGCAATCCATCAG		
F. proliferatum	Fp3-F	CGGCCACCAGAGGATGTG	230	Jurado et al. 2006
	Fp4-R	CAACACGAATCGCTTCCTGAC		
F. culmorum	Fc01F	ATGGTGAACTCGTCGTGGC	570	Nicholson et al. 1998
	Fc01R	CCCTTCTTACGCCAATCTCG		
F. graminearum	Fg16F	CTCCGGATATGTTGCGTCAA	450	Nicholson et al. 1998
	Fg16R	GGTAGGTATCCGACATGGCAA		
F. sporotrichioides	AF330109CF	AAAAGCCCAAATTGCTGATG	332	Demeke et al. 2005
	AF330109CR	TGGCATGTTCATTGTCACCT		

Table 2 Primers used for DNA sequencing.

Primer name	Sequence (5'−3')	Source
EF1	ATGGGTAAGGARGACAAGAC	O'Donnell et al. 1998
EF2	GGARGTACCAGTSATCATGTT	
BT2A	GGTAACCAAATCGGTGCTGCTTTC	Glass and Donaldson 1995
BT2B	ACCCTCAGTGTAGTGACCCTTGGC	
ITS1	TCCGTAGGTGAACCTGCGG	White et al. 1990
ITS4	TCCTCCGCTTATTGATATGC	

Table 3 PCR conditions used.

PCR assay	Initial denaturation [a]	Denaturation [b]	Annealing [b]	Extension [b]	Final extension [a]	Source
F. verticillioides	94 °C/2 min	95 °C/35 s	64 °C/30 s	72 °C/2 min	72 °C/5 min	Patiño et al. 2004
F. proliferatum	94 °C/5 min	95 °C/30 s	69 °C/45 s	72 °C/45 s	72 °C/10 min	Jurado et al. 2006
F. culmorum, F. graminearum, F. sporotrichioides	95 °C/3 min	95 °C/30 s	62 °C/20 s	72 °C/45 s	72 °C/5 min	Demeke et al. 2005
Elongation factor	94 °C/5 min	94 °C/30 s	53 °C/30 s	72 °C/45 s	72 °C/10 min	O'Donnell and Cigelnik, 1997
β-tubulin	94 °C/5 min	94 °C/30 s	60 °C/45 s	72 °C/1 min	72 °C/7 min	Glass and Donaldson, 1995
ITS	94 °C/5 min	94 °C/30 s	50 °C/30 s	72 °C/45 s	72 °C/10 min	White et al. 1990

[a] Conducted in one cycle.

[b] Conducted in 35 cycles (*F. verticillioides*, *F. proliferatum*, Elongation factor, β-tubulin and ITS) and 38 cycles (*F. culmorum*, *F. graminearum* and *F. sporotrichioides*).

Table 4 Water activity (a_w) and moisture content (m.c., in %) after harvest in control and high-carotenoid maize during the trials. Means within a column with no superscript in common are significantly different ($p < 0.05$).

	2013		2014		2015	
	a_w	m.c.	a_w	m.c.	a_w	m.c.
Control maize	0.893 [b]	23.15 [b]	0.954 [a]	30.13 [a]	0.968 [a]	41.59 [a]
High-carotenoid maize	0.921 [a]	26.83 [a]	0.954 [a]	27.20 [b]	0.972 [a]	42.06 [a]

Fig. 1 Meteorological data (temperature and rainfall) from May to November in the experimental field in Lleida (Catalonia, Spain) in 2013, 2014 and 2015.

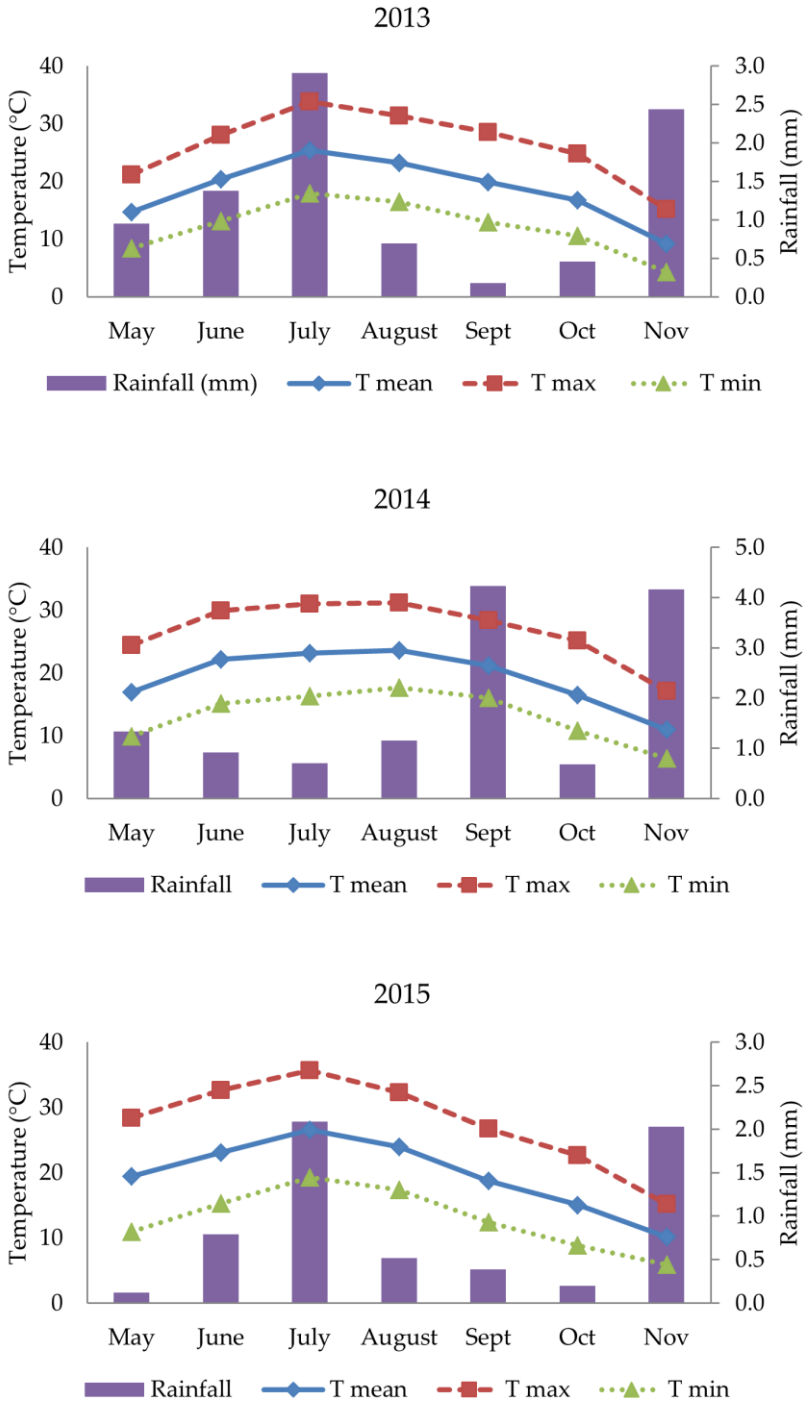

Fig. 2 Mycobiota identification in control and high-carotenoid maize during the trials.

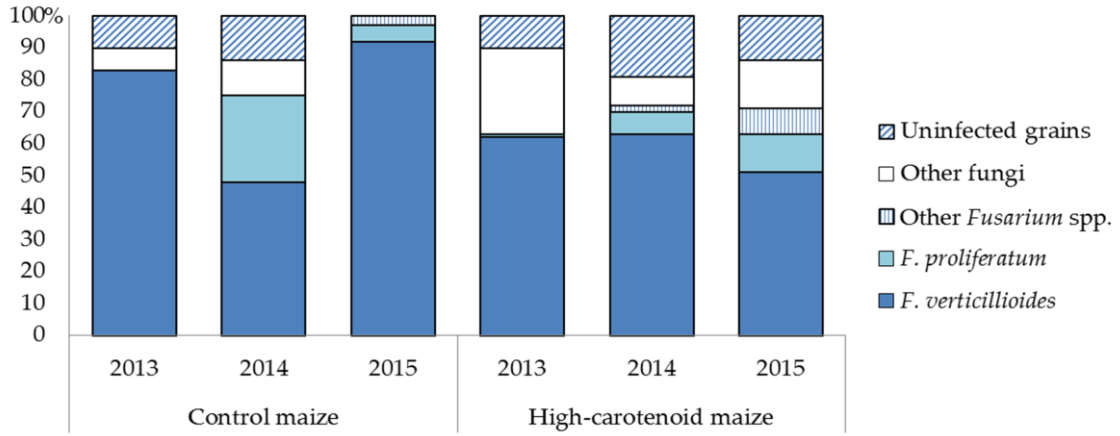

Fig. 3 Fumonisin levels (FB_1 + FB_2, in mg/kg) in control and high-carotenoid maize during the trials. The values shown are the mean and standard error for each treatment (n=3). The asterisk indicates statistically significant differences between types of maize ($p < 0.05$).