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Article

Influence of storage environment on maize grain: CO_2 production, dry matter losses and aflatoxins contamination

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Abstract

Poor storage of cereals such as maize can lead to both nutritional losses and mycotoxin contamination. The aim of this study was to examine the respiration of maize either naturally contaminated or inoculated with *Aspergillus flavus* to examine whether this might be an early and sensitive indicator of aflatoxin contamination and relative storability risk. We thus examined the relationship between different interacting storage environmental conditions (0.80-0.99 water activity (a_w) and 15-35°C) in naturally contaminated and irradiate maize grain + *A. flavus* on relative respiration rates (R), dry matter losses (DMLs) and aflatoxin B1 and B2 (AFB1-B2) contamination. Temporal respiration and total CO_2 production were analysed by GC-TCD, and results used to calculate the DMLs due to colonisation. Aflatoxins (AFs) contamination were quantified at the end of the storage period by HPLC MS/MS. The highest respiration rates occurred at 0.95 a_w and 30-35°C representing between 0.5-18% DMLs. Optimum AFs contamination was at the same a_w at 30°C. Highest AFs contamination occurred in maize colonised only by *A. flavus.* A significant positive correlation between %DMLs and AFB1 contamination was obtained ($r=0.866$, $p<0.001$) in the irradiated maize treatments inoculated with *A. flavus*. In naturally contaminated maize + *A. flavus* inoculum loss of only 0.56% DML resulted in AFB_1 contamination levels exceeding the EU legislative limits for food. This suggests that there is a very low threshold tolerance during storage of maize to minimise

AFB_1 contamination. This data can be used to develop models which can be effectively used in enhancing management for storage of maize to minimise risks of mycotoxin contamination.

Keywords

Cereals; corn; temperature; water activity; *Aspergillus flavus*; mycotoxins; carbon dioxide; silos

1 Introduction

Maize (*Zea mays* L.), also called corn, is an annual grass in the family Poaceae and a staple food crop grown all over the world. World maize production increased from 272 to 1,060 million tonnes from 1967 to 2016 growing at an average annual rate of 3.20 % (KNOEMA, n.d.). As the world's population increases, demand for maize in developing countries is expected to double by 2025. This higher demand also includes a large variety of food and industrial maize-based products, as well as maize for animal feed (Gryseels et al., 2015).

As maize is a basic staple component of the diet in many regions of the world, its production needs to be maintained at high standards in terms of sensorial, nutritional and microbiological quality. However, nutritional and dry matter losses (DMLs) can often be caused by spoilage moulds and contamination with mycotoxins during pre- and post-harvest phases (Magan & Aldred, 2007). The main fungal species and mycotoxins associated with maize are *Aspergillus flavus* and aflatoxins (AFs), *Fusarium verticillioides* and *F. proliferatum* and fumonisins (FMs), *F. graminearum* and trichothecenes (TCT) and zearalenone (ZEA) (Chulze, 2010).

Aflatoxin B1 (AFB1) is a naturally produced toxin that can cause cancer in animals and human beings (IARC, 2002). For this reason, there are strict legislative limits for the maximum contamination of maize with AFB1 and for total AFs. According to the EU, which has the strictest limits worldwide, the maximum allowable AFB1 is 5 $\mu g kg^{-1}$ in raw commodities (maize, tree nuts and groundnuts); 2 $\mu g\ kg^{-1}$ in processed food commodities (Commission, 2006) and 20 $\mu g\ kg^{-1}$ for feed (Commission, 2003).

A. flavus can infect maize at both pre- and post-harvest stages and an increase in aflatoxin content can occur when the drying phase and storage are poorly managed. Maize is generally harvested at a relatively high moisture content (m.c.) of 19-22% (Seitz, Sauer, Mohr, & Aldis, 1982). Once moist grain is harvested, the grain is dried and stored in silos for the medium or long-term (Kaleta & Górnicki, 2013; Magan & Aldred, 2007). If maize is stored safely (14.5-

15% m.c. = 0.70 a_w) no moulds can grow and the grain has a basal rate of respiration. However, the activity of pests can result in the accumulation of moisture resulting in the initiation of spoilage fungal growth (Chulze, 2010). Metal silos are affected by the weather conditions and can become damp internally from condensation on their sidewalls caused by changes in humidity and temperature. This moisture can be transferred to the stored commodity in the silo providing ideal conditions for fungal proliferation and mycotoxin accumulation.

When the m.c. increases, both the respiration of the grain and that of the associated mycobiota increases. This results in utilisation of the grain nutrients by the spoilage fungi resulting in deterioration of quality and associated DMLs (Seitz et al., 1982). Saul & Lind (1958) first attempted to correlate the impact of elevated CO_2 and DML on fungal growth and mycotoxin production. According to Seitz et al., (1982), the contribution to DML from fungi increases during storage at a rate dependent on moisture, temperature, amount and type of kernel damage and level of fungal inoculum on the grain. Recent studies have examined the use of CO_2 production during storage of maize, wheat and rice as an indicator of the level of AFs, FMs, deoxynivalenol (DON), ZEA and trichothecenes A (TCT-A) contamination (Garcia-Cela, Kiaitsi, Medina, et al., 2018; Martín Castaño, Medina, & Magan, 2017a,b; Mylona & Magan, 2011; Mylona, Sulyok, & Magan, 2012) These studies proved that it is possible to utilise the progressive increase in the respiration rate under increasingly conducive conditions for mould growth due to the oxidation of carbohydrates and hence CO_2 production, water vapour and heat during aerobic respiration to calculate quality losses as DML. DML can be quantified based on CO_2 production and respiration rates using Gas Chromatography (GC) and these data sets are used as a "storability risk index" to predict overall quality changes in stored grain.

Previously, DML was used as a grain quality indicator. Values as low as 0.04% DML were considered to have an impact on seed germination and on early moulding of wheat (Lacey, Hamer, & Magan, 1994; White, Sinha, & Muir, 1982). Seitz et al., (1982) showed that a loss of 0.5% DML in stored maize was enough to downgrade this commodity from food to feed, with associated increased risks of aflatoxin contamination. DML of between 1 and 2% in cereals (rice, wheat, maize) contaminated with *Fusarium* toxins (FMs, DON and ZEA) resulted in contamination levels which exceeded the EU legislative limits (Garcia-Cela, Kiaitsi, Medina, et al., 2018; Martin Castaño, Medina, & Magan, 2017a,b; Mylona, Sulyok, & Magan, 2012). Indeed, DMLs of <1% in oats and rice contaminated with AFB_1 and other trichothecene (T-2/HT-2) toxins exceed the EU legislative limits (Martin Castaño, Medina, & Magan, 2017a; Mylona & Magan, 2011). This suggests that CO_2 production could be a powerful tool for the

early prediction of the level of contamination of the grain with mycotoxins (Mylona, Sulyok, & Magan, 2012).

The objectives of this study were to examine the effect of storage temperature (T) x water activity (a_w) conditions (15–35°C; 0.80–0.99 a_w) of naturally contaminated, and gamma irradiated stored maize, and these inoculated with *A. flavus* on: (a) respiration rate (R), (b) total cumulative CO_2 production, (c) DML% in the stored maize treatments, (d) quantification of AFB_1 and AFs contamination levels in the different treatments, and (e) determination of the relationship between DML and AFB1 contamination to identify storage conditions which represent a low and high risk of AFs contamination of maize during storage.

2 Material and Methods

2.1 Fungal isolate

An aflatoxigenic type strain of *A. flavus* (NRRL 3357; Northern Regional Research Laboratories (NRRL) of the US Department of Agriculture USDA, New Orleans) was used in this experiment. The strain was maintained in glycerol:water (70:30, v/v) at -20°C in the culture collection of the Applied Mycology Group, Cranfield University.

2.2 Maize samples treatment, moisture content and water activity adjustment

Two batches of feed-grade maize grain derived from France were used. One batch was naturally contaminated maize for storage experiments; the 2nd batch was gamma irradiated (12-15 kGys; SynergyHealth, Swindon, U.K.) in order to disinfect the grain from any fungal contaminants while retaining germinative capacity (Magan, Aldred, Mylona, & Lambert, 2010). The mycobiota and the germination of the maize was checked. Fifty naturally contaminated and 50 irradiated maize kernels were placed, 5 per 9 cm Petri plate containing Malt Extract Agar (MEA), in a sterile flow bench, and then incubated at 25°C for 7 days. After this period the fungal contamination was evaluated. In addition, 5 x 10 maize kernels of each type were placed on 9 cm Petri dishes containing moist filter paper. The a_w of the maize batches was about 0.70 a_w. Both batches were stored at 4°C in re-sealable polyethylene bags until use in experiments.

2.3 Development of the moisture adsorption curves

Separate moisture adsorption curves were developed for both naturally contaminated maize and the irradiated maize. To this end, 10 g sub-samples were placed in 25 mL Universal glass

bottles and known amounts of water were added. Replicate samples were sealed and stored at 4°C for 24 h with regular shaking. The samples were then equilibrated at 25°C and the a_w and moisture content (m.c.) were determined. The a_w was measured using an AquaLAB Water Activity Meter 4 TE (Decagon Devices, Inc, Pullman, USA) at 25°C. The moisture content (m.c., wet weight basis) was determined by drying at 105°C for 16 h. The amounts of added water were plotted against a_w levels to accurately modify the stored maize treatments to the target a_w levels. The relationship between the m.c. and the a_w was also plotted for reference purposes.

2.4 Grain inoculation and incubation

The *A. flavus* strain was sub-cultured on 3% milled maize meal agar (1.5%) medium (MMA) on 9 cm Petri plates and incubated at 25°C for 7 days to obtain heavily sporulating cultures. A sterile loop was used to remove the conidia which were suspended in 10 mL sterile water containing 0.005% Tween 80. After vigorous shaking to obtain a spore suspension the concentration was quantified using a Thoma counting chamber (Marienfield) and the suspension adjusted to a final concentration of 1×10^5 spores mL^{-1} in sterile water + 0.005% tween 80.

For storage experiments maize grain (10 g) were modified to different target a_W levels with sterile water (0.80, 0.85, 0.90, 0.95 and 0.99 a_W) and equilibrated as detailed previously in 40 mL vials (Chromacol Ltd, UK) with sealable caps provided with a septum for gas removal. A known amount of sterile water except for 10 µL were added aseptically to each vial in order to reach the a_w target and equilibrated at 4°C for 24 h. After this, 10 µL of 1×10^5 spores mL^{-1} were added to the inoculated or control samples respectively and shaken by hand for 10 s. Vials with the same a_w were enclosed in 16 L containers also containing glycerol-water solutions (1 L) to maintain the equilibrium relative humidity (ERH) of the atmosphere at the target a_w level of the treatment and sealed. The replicates and treatments were stored at 15°C/0.99 a_W; and 20, 25, 30, 35°C/0.80, 0.85, 0.90, 0.95 a_W). For each condition, four replicates per treatment were used.

2.5 Respiration of maize grain stored under different a_w x temperature conditions

Carbon dioxide (CO_2) production were measured on alternate days (1, 3, 5, 7, 9, and 11 days). The sampling method used was as previously described by Mylona & Magan (2011). However, the specific volume of head space was considered. For calculating the head-space, vials containing the different water activity modified grain were filled with water and the volumes

necessary immediately measured. The head-space volumes were 34, 33, 32 and 29 mL for 0.90, 0.93, 0.95 and 0.99 a_w treatments respectively.

Vials were sealed under sterile conditions and stored for 1 h at the treatment conditions before CO_2 was removed. Five mL of the headspace were withdrawn, and 2 mL were directly inserted into the sampling chamber of the GC for CO_2 analysis. The GC equipment used was an Agilent 6890N Network Gas Chromatograph (Agilent Technologies, UK) with a Thermal Conductivity Detector (TCD) and helium as a carrier gas. The column used for the analyses was packed with Chromosorb 103 and the data analysed using the Agilent Chemstation Software (Agilent Technologies, UK). A calibration standard was used of 10.06% CO_2, 2% O_2 in nitrogen (BOC cylinder).

The percentages of CO_2 concentration were used to calculate (a) Respiration (R) rate in mg CO_2 (kg h)$^{-1}$, (b) total cumulative production of CO_2 after 11 days storage and (c) the total Dry Matter Losses (DMLs; (Mylona & Magan, 2011).

2.6 Mycotoxin analysis

2.6.1 Sample preparation

Maize grain was dried at 60°C for 48 h, milled and stored at 4°C pending further analysis. Five grams of milled maize were extracted using 20 mL extraction solvent (acetonitrile/water/acetic acid 79/20/1) followed by a 1+1 dilution using acetonitrile/water/acetic 79/20/1. Five µL of the diluted extract were directly injected into the sampling port for LC-MS/MS analysis.

2.6.2 LC-MS/MS parameters

LC-MS/MS screening of targeted fungal metabolites was performed with a QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25°C on a Gemini® C_{18}-column, 150 x 4.6 mm i.d., 5 µm particle size, equipped with a C_{18} 4 x 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, US). The chromatographic method as well as chromatographic and mass spectrometric parameters were previously described (Malachová, Sulyok, Beltrán, Berthiller, & Krska, 2014).

ESI-MS/MS was performed in the time-scheduled multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. The MRM detection window of each analyte

was set to its expected retention time ± 27 and ± 48 seconds in the positive and the negative mode, respectively.

Quantification was performed via external calibration using serial dilutions of a multi-analyte stock solution. The limit of detection was 0.6, 0.6, 4.1 and 10 µg/kg for AFB1, AFB2, AFG1 and AFG2, respectively. The validated recoveries were 73%. The accuracy of the method has been verified on a continuous basis by regular participation in proficiency testing schemes (Malachova, Michael, Beltran, Berthiller, & Krska, 2015; Malachová et al., 2014).

2.7 Statistical Analysis

Statistical analysis was performed using the package JMP® Pro 13 (SAS Institute Inc., 2016. Cary, NC, USA). Datasets were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene test, respectively. When data failed the normality test, variable transformation was performed to try to improve normality or homogenise the variances. Transformed data were still not normally distributed and therefore the Wilconxon or Kruskal-Wallis test by ranks was used for the analysis of the data. Nonparametric comparisons for each pair using the Wilcoxon Method were used to find differences between groups.

For statistical analysis LOD/2 was considered when samples were <LOD.

Forward stepwise regression was used to obtain polynomial equations for Log_{10}DML with regard to the storage conditions (a_w and T). The assumptions of linearity and normally distributed residuals were assessed, producing normal plots of the residuals. Contour maps were built in JMP® Pro 13 using 5000 simulation data from predicted formula.

3 Results

3.1 Effect of a_w and temperature on the temporal respiration rates of *A. flavus* when colonising maize and the accumulated total CO_2 production

Figure 1 and 2 show the temporal respiration activity (hourly) in natural or irradiated maize inoculated with *A. flavus* at 30°C and the total accumulated CO_2 (cumulative R; g CO_2 kg maize^{-1}) at 5 different a_w levels. Similar data was obtained over the temperature range of 15-35°C in grain stored for 11 days.

Overall, respiration in both natural and irradiated stored maize at 0.80-0.85 a_w was consistently low, regardless of the storage temperature. The highest respiration rates were recorded at 0.95 a_w for natural (1387 mg CO_2 kg^{-1} h^{-1}) and irradiated maize grain (698 mg CO_2 kg^{-1} h^{-1})

inoculated with *A. flavus* at 35°C (data no shown) and this was confirmed by the total accumulated CO_2 production.

Respiration rates in samples inoculated with *A. flavus* generally started to increase after 3 days of storage depending on the a_w x temperature conditions used. CO_2 production was higher in natural maize grain, compared to the irradiated treatments (ChiSquare *p<0.0001*). Statistically differences were found in both natural and irradiated maize grain between treatments having no additional inoculum of *A. flavus* and those with an inoculum (ChiSquare *p<0.0001*). This was particularly pronounced in the irradiated maize treatments (Figure 2). The background respiration rates measured in irradiated maize grain were generally very low in all conditions tested.

3.2 Effect of storage of maize treatments on dry matter losses

Based on the total accumulated CO_2 production, the DMLs of naturally contaminated maize grain and that inoculated with *A. flavus* under all the tested conditions were quantified in Figure 3. This shows that DMLs in both natural and irradiated maize increased significantly with increasing a_w and temperature conditions (ChiSquare *p<0.0001*) (Suppl. Table A). Inoculation with *A. flavus* always resulted in a much higher amount of DML in all treatments. The only exception was for naturally stored maize at 20°C/0.95 a_w and 30°C/0.90 a_w. The highest % DMLs was observed at 0.95 a_w in the samples inoculated with *A. flavus* (up to 17%).

A polynomial model ($Log_{10}DML=b_0+b_1T+b_2a_w+T^2b_3+a_w^2b_4+Txa_wb_5$) (Eq.1) was obtained by forward stepwise regression for the effect of the storage conditions on the Log_{10} transformed data of DMLs in natural maize and that inoculated with *A. flavus.* The interaction was not significant and therefore was not included in the model. The values for the coefficients b_0-b_4 as well the statistical significance of the factors in each case are presented in Table 1.

Figure 4 shows contour maps for the relationship between a_w x T and optimum and marginal conditions for DMLs in naturally contaminated maize and that inoculated with *A. flavus*. For example, at 25°C and 0.90a_W there was a much higher level of DML in maize grain + *A. flavus* inoculum (5.1%) when compared to naturally contaminated maize (0.63% DML).

3.3 Aflatoxins production in wheat and maize under different storage conditions

The analyses method allowed the quantification of all four aflatoxins (B1, B2, G1, G2). However, AFG1 was only detected in one sample of natural maize at 25°C/0.95 a_W, while AFG2 was never detected. Therefore, only AFB1 and AFB2 were examined for statistical analysis. Table 2 shows the AFB1 and AFB2 data from naturally stored maize grain, as well

as in the natural or irradiated maize grain treatments inoculated with *A. flavus.* AFB1 contamination represented >85% of the total AFs in positive samples. A_w significantly affected the AFB1 contamination (ChiSquare *p<0.0001*) in all the treatments analysed (see Suppl. Table B). Similar trends were observed with data for AFB2. In general, the highest content of AFs were detected in the wettest grain treatment (0.95 a_W). The only exception was at 35°C in natural maize where a peak of production was detected at 0.80 a_W. Although the T was not a significant factor probably due to the higher variation between samples of the same treatments. Overall, the optimum temperature range for toxin production was between 25 and 35°C.

3.4 Correlation between DMLs and AFB_1 and AFs contamination relevant to EU legislative limits

AFB_1 data was plotted against DMLs for natural maize, and natural maize + *A. flavus* in Figure 5. Indicator lines depicting the EU legislative limits for AFs in feed materials (AFB1: 20 µg kg^{-1}) and maize for human consumption or use as an ingredient in food (AFB1: 5 µg kg^{-1}) (Commission, 2003, 2006) have been added for a better understanding of the relevance. Most of the analysed samples that contained AFB1 below the legal limits occurred under marginal conditions of temperature and moisture for growth of *A. flavus*. Although Spearman correlations were significant, only higher r^2 correlation was obtained with irradiate maize treatments inoculated with *A. flavus* (r^2=0.8660). This is probably due to the natural maize having a mixed mycobiota, many of which may be actively growing but are not aflatoxin producers. However, it is clear that higher DMLs could indicate higher probabilities of AFB1 contamination of the stored maize. From our results different DMLs limits could be established as a control limit in relation to the kind of matrix studied. Almost all positive results were above the legal limits for food and feed in naturalmaize + *A. flavus* inoculum where this occurred at a very low DML of only 0.56% [DML_{log10} (-0.25)]. In the case of irradiated maize + *A. flavus* even smaller losses in dry matter [0.30% DML (DML_{log10} (-0.5)] would result in contamination being above the legislative limits.

4 Discussion

Different abiotic parameters (time, a_w and temperature) were tested in this study to determine the CO_2 production of natural and irradiated maize grain with the associated mycobiota and with *A. flavus* inoculation respectively. The highest respiration and total cumulative CO_2 production rates were observed at 30-35°C in the wettest conditions (0.95 a_w) tested throughout the storage period. This allowed the calculation of the % DMLs under different

interacting a_w x temperature storage conditions (Mylona & Magan, 2011). In parallel, DMLs for both types of maize grain appeared to increase with increasing a_w and temperature conditions. The maximum % DML of about 17.11% was obtained at 35°C with natural maize inoculated with *A. flavus*. Most previous studies on maize colonised by *A. flavus* were carried out over a limited temperature x a_w range of 25-30°C and 0.90-0.99 a_w (Bluma & Etcheverry, 2008; Garcia, Ramos, Sanchis, & Marín, 2013; Nesci, Gsponer, & Etcheverry, 2007). The only exception was that Samapundo et al. (2007) tested a wider range of storage conditions between 16-37°C and 0.80-0.98 a_W. They observed no growth of their strain of *A. flavus* at 37°C with maximum growth at 30°C. However, none of these studies found interactions between a_w and 35°C. Indeed, recent studies of the impact of interacting climate change factors of a_w x temperature and elevated CO_2 have also suggested that both growth and AFs production occur at 37°C (Medina, Gilbert, Mack, Obrian et al., 2017).

It is worth noting that there were significant differences between natural and irradiated maize samples. Naturally contaminated maize samples showed higher respiration rates and DMLs, regardless of storage conditions. This may be explained by the initial mycobiota in the maize grain, which was eliminated during the irradiation process. The main fungal genera were *Rhizopus, Mucor, Penicilium* spp. and *Aspergillus* section *Flavi* (data not shown). This suggests that the mycobiota present was a good representation of the fungal community in maize entering storage and thus the data sets from the present study would be beneficial to a better understanding of the potential for maize spoilage and mycotoxin contamination. The data could also be a basis for the development of a database and model which can be utilised for examining risks of toxin contamination in grain silos.

The relationship between CO_2 and storage conditions allowed the calculation of DMLs due to colonisation by spoilage fungi. While common mycobiota of maize includes toxigenic species within the *Aspergillus* section *Flavi* species (aflatoxin producers) or *Fusarium* section *Liseola* (e.g. *F. verticilloides,* fumonisin producer), other non-mycotoxigenic species can result in significant nutritional quality losses and thus have economic impacts. The increased % DMLs obtained over time and temperature in this study can be correlated with the results of Gailliez (2013). This previous study investigated the relationship between nutritional value of maize kernels in terms of total carotenoids and β-carotene and fungal contamination. The results showed a significant decrease of thiamine content in maize contaminated with *A. flavus* in the wettest conditions examined (Gailliez, 2013).

Regarding AFs production, the highest contamination levels were detected in the wettest grain treatment tested (0.95 a_W). Overall, the optimum temperature range for production in our study

was between 25-35°C. In general, the highest AFs production was observed when *A. flavus* grew alone (irradiated stored maize). The only exception was at 35°C in natural maize where a peak of production was also detected at 0.80 a_W. Astoreca, Vaamonde, Dalcero, Marin, & Ramos (2014) examined *A. flavus* colonisation of maize-based media over a wide range of environmental conditions (10-40°C vs 0.80-0.98 a_W) and found optimum AFB1 at 0.96 a_W and 30°C.

In the present study the treatments with natural mycobiota or mycobiota + *A. flavus* inoculum better represented the conditions which may occur under low or high contaminated batches of maize grain with potentially toxigenic contaminants. This showed that DMLs were slightly higher (18 vs 15-16%) in the natural maize + *A. flavus* inoculum than without the inoculum. These results could be explained by the artificial increase on the total number of microorganisms present in the maize due to the *A. flavus* inoculation.

Previously, inoculation of maize with mixed species resulted in a reduction in AFB1 concentration. Thus, co-cultures of *A. flavus* and *P. purpurogenum* in maize showed the lowest production, while that inoculated with *A. flavus* alone (control) resulted in the highest contamination levels (Oyebanji & Efiuvwevwere, 1999). Other studies with co-inoculation of irradiated maize grains with *A. flavus* and *F. proliferatum* resulted in an inhibition of AFB1 production at 0.97 a_w and 25°C (Picco, Nesci, Barros, Cavaglieri, & Etcheverry, 1999). Indeed, the ecological niches occupied by these two species are different and the observed effect might be explained by a switch between *Fusarium* and *A. flavus* colonisation depending on a_w x temperature conditions with >0.95 a_w and 25-30^{o}C favouring *Fusarium* growth and hence fumonisin contamination. Conversely, under drier and warmer conditions (30-35^{o}C) growth of and AFs contamination would be supported (Giorni, Magan, Pietri, Bertuzzi & Battilani, 2007)

Another example, where mixed inoculums of *F. culmorum* and *A. carbonarius* were used, the impact on DON and OTA production was very different. For *F. culmorum*, the presence of other species often inhibited DON production over a range of environmental conditions. For *A. carbonarius*, on a grape-based medium the presence of certain species resulted in a significant stimulation of OTA production (Magan, Aldred, Hope & Mitchell, 2010).

It is worthwhile mentioning that the DMLs should not only be related to initiation of mould spoilage, but also as an indicator of potential toxin contamination and classification as being either for human consumption or for animal feed. Our results have shown that the maximum DMLs (15-18%) corresponded to high contamination levels with AFB1, which were above the EU legislative limits for both food and feed maize (5 µg kg^{-1} and 20 µg kg^{-1} respectively) (Commission, 2003, 2006). Indeed, the present study suggests that at approx. 0.56 % DML in

maize contaminated with *A. flavus* may represent an increased risk of AFB_1 contamination levels being above the legislative limits for food. Anything higher than this would potentially represent a very high risk of contamination with this carcinogenic toxin.

A comparison with previous studies on oats colonised by *F. langsethiae* (T-2, HT-2 toxins), maize *and F. verticillioides* (fumonisins), wheat and *F. graminearum* (ZEA and related toxins) and paddy and brown rice colonised by *A. flavus* and *F. verticillioides* (Garcia-Cela, Kiaitsi, Medina, Magan, 2018; Martin Castaño, Medina, & Magan, 2017a,b; Mylona & Magan, 2011; Mylona, Sulyok, & Magan, 2012) could be made. In these previous studies, DML of between 1 and 2% in cereals (rice, wheat, maize) contaminated with *Fusarium* toxins (FMs, DON and ZEA) resulted in levels exceeding the EU legislative limits. Indeed, DMLs of <1% in oats and rice contaminated with AFB^1 and T-2/HT-2 toxins exceeded the EU legislative limits for this toxin. DMLs could be also an indicator in hazelnuts where only 0.4% can cause aflatoxin problems (Mylona, 2012). Consequently, a relationship exists between small DMLs and the potential risk of exceeding legislative limits especially for human consumption. Intermediate tolerances to DMLs may be possible for commodities destined for animal feed use (Garcia-Cela, Kiaitsi, Medina, Magan, 2018).

This study suggests that CO_2 production data could be used as an early indicator of the initiation of fungal or indeed pest activity which can be linked to the potential for mycotoxin production and more importantly the relative level of the risk of exceeding the existing EU legislation for food and feed. We are now examining the use of these types of datasets to build models which can be coupled with real time data collection on CO_2 production in storage facilities, especially grain silos of stored grain to develop an effective tool for better/improved management of stored commodities post-harvest. This would have the benefits of minimising the risk of mould spoilage and mycotoxin contamination and allow remedial action to be taken rapidly should this be required.

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Figure 2: Temporal CO_2 production (R) and accumulation (R cumulative) obtained from GC measurements of irradiated maize alone and that inoculated with *A. flavus* at 30ºC. Vertical bars represent standard error of the mean.

Figure 3: Percentage of DMLs in stored maize inoculated with *A. flavus* at different a_w x temperature conditions for 11-days. Vertical bars represent standard error of the mean.

Figure 4. DML contour maps describing the DMLs in natural grain and irradiated maize inoculated with *A. flavus* under different combinations of environmental conditions.

Figure 5: Scatter plot of DMLs and AFB1 in stored maize after 11 days storage under all the environmental conditions examined producing in natural maize by a) natural mycobiota, b) natural mycobiota + *A. flavus* and in irradiate maize grain by c) *A. flavus*. Horizontal lines represent legal European limits. Nonparametric Spearmans correlation Elipse α=0.95.

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References

Astoreca, A., Vaamonde, G., Dalcero, A., Marin, S., & Ramos, A. (2014). Abiotic factors and their interactions influence on the co-production of aflatoxin B1 and cyclopiazonic acid by *Aspergillus flavus* isolated from corn. *International Journal of Food Microbiology*, *38*, 276–283. https://doi.org/10.1016/j.fm.2013.07.012

Bluma, R. V., & Etcheverry, M. G. (2008). Application of essential oils in maize grain: Impact on *Aspergillus* section *Flavi* growth parameters and aflatoxin accumulation. *Food Microbiology*, *25*(2), 324–334. https://doi.org/10.1016/J.FM.2007.10.004

Chulze, S. N. (2010). Strategies to reduce mycotoxin levels in maize during storage: a review. *Food Additives & Contaminants: Part A*, *27*(5), 651–657. https://doi.org/10.1080/19440040903573032

Commission, E. (2003). Commission Directive 2003/100/EC of 31 October 2003 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed (Text with EEA relevance). *Official Journal L 285 , 01/11/2003 P. 0033 - 0037;*, *L285*, 0033–0037. Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32003L0100&from=EN

Commission, E. (2006). Commission Regulation (EC) No. 1881/2006 of 19 December 2006, Setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*, *364*, 5–24. Retrieved from http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0005:0024:EN:PDF

Gailliez, A. (2013). Effect of different storage conditions on the nutritional value of maize contaminated with *Aspergillus flavus* and *Fusarium verticillioides*. Doctoral Thesis Cranfield University.

Garcia-Cela, E., Kiaitsi, E., Sulyok, M., Medina, A., & Magan, N. (2018). *Fusarium graminearum* in stored wheat: Use of CO_2 production to quantify dry matter losses and relate this to relative risks of zearalenone contamination under interacting environmental conditions. *Toxins*, *10*(2). https://doi.org/10.3390/toxins10020086

Garcia, D., Ramos, A. J., Sanchis, V., & Marín, S. (2013). Modeling kinetics of aflatoxin production by *Aspergillus flavus* in maize-based medium and maize grain. *International Journal of Food Microbiology*, *162*(2), 182–189. https://doi.org/10.1016/J.IJFOODMICRO.2013.01.004

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., & Battilani, P. (2007). Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy. *International Journal of Food Microbiology*, *113*(3), 330–338. https://doi.org/10.1016/j.ijfoodmicro.2006.09.007

Gryseels, G., Betran, J., Chapman, S., Ketema, S., Schdeva, P., & Snapp, S. (2015). Evaluation of the CGIAR Research Program on MAIZE *Volume 1-Evaluation Report*. Retrieved from http://iea.cgiar.org/wp-content/uploads/2016/10/Maize-Volume-1.Final_.pdf

IARC. (2002). IARC Monographs on the evaluation of carcinogenic risks to humans some traditional herbal medicines, some mycotoxins, naphthalene and styrene. (World Health Organization, Ed.) (Volume 82). Lyon. Retrieved from https://monographs.iarc.fr/wp-content/uploads/2018/06/mono82.pdf

Kaleta, A., & Górnicki, K. (2013). Criteria of determination of safe grain storage time. In S. Grundas (Ed.), *A Review, Advances in Agrophysical Research*. InTech.

KNOEMA. (n.d.). World Maize production, 1961-2017 - knoema.com. Retrieved July 14, 2018, from https://knoema.com/atlas/World/topics/Agriculture/Crops-Production-Quantity-tonnes/Maize-production?origin=knoema.es&_ga=2.268320334.1514206023.1531579067-305608684.1531579067

Lacey, J., Hamer, A., & Magan, N. (1994). Respiration and dry matter losses in wheat grain under different environmental factors. In E. Highley, E. J. Wright, H. J. Banks, & B. R. Champ (Eds.), *In Stored Product Protection* (pp. 1007–1013).

Magan, N., & Aldred, D. (2007). Post-harvest control strategies: Minimizing mycotoxins in the food chain. *International Journal of Food Microbiology*, *119*(1–2). https://doi.org/10.1016/j.ijfoodmicro.2007.07.034

Magan, N., Aldred, D., Mylona, K., & Lambert, R. J. W. (2010). Limiting mycotoxins in stored wheat. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *27*(5). https://doi.org/10.1080/19440040903514523

Magan, N., Aldred, D., Hope, R., & Mitchell, D. (2010). Environmental factors and interactions with mycobiota of grain and grapes: effects on growth, deoxinivalenol and ochratoxin production by *Fusarium culmuron* and *Aspergillus carbonarius*. *Toxins*, *27*(5). https://www.sciencedirect.com/science/article/pii/S0168160517302222

Malachová, A., Sulyok, M., Beltran, E., Berthiller, F., & Krska, R. (2015). Multi-Toxin Determination in Food – The Power of Dilute and Shoot; Approaches in LC–MS–MS. Retrieved from http://www.chromatographyonline.com/multi-toxin-determination-food-power-dilute-and-shoot-approaches-lc-ms-ms

Malachová, A., Sulyok, M., Beltrán, E., Berthiller, F., & Krska, R. (2014). Optimization and validation of a quantitative liquid chromatography–tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices. *Journal of Chromatography A*, *1362*, 145–156. https://doi.org/10.1016/j.chroma.2014.08.037

Martín Castaño, S., Medina, A., & Magan, N. (2017). Comparison of dry matter losses and aflatoxin B1 contamination of paddy and brown rice stored naturally or after inoculation with *Aspergillus flavus* at different environmental conditions, *Journal of Stored Products Research 73*, 47–53. https://doi.org/10.1016/j.jspr.2017.06.004

Martin Castaño, S. , Medina, A., & Magan, N. (2017). Impact of storage environment on respiration, dry matter losses and fumonisin B_1 contamination of stored paddy and brown rice. *World Mycotoxin Journal*, *10*(4), 319–326. https://doi.org/10.3920/WMJ2017.2237

Medina, A., Gilbert, M.K, Mack, B.M., Obrian, G., Rodriguez, A., Bhatnagar, D., Payne, G., Magan, N., 2017. Interactions between water activity and temperature on the *Aspergillus flavus* transcriptome and aflatoxin B1 production. *International Journal of Food Microbiology*, 256, 36-44. https://www.sciencedirect.com/science/article/pii/S0168160517302222.

Mylona, K., & Magan, N. (2011). *Fusarium langsethiae*: Storage environment influences dry

matter losses and T2 and HT-2 toxin contamination of oats. *Journal of Stored Products Research*, *47*(4), 321–327. https://doi.org/10.1016/j.jspr.2011.05.002

Mylona, K., Sulyok, M., & Magan, N. (2012). Relationship between environmental factors, dry matter loss and mycotoxin levels in stored wheat and maize infected with *Fusarium* species. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *29*(7), 1118–28. https://doi.org/10.1080/19440049.2012.672340

Mylona, K. (2012). *Fusarium* species in grains: dry matter losses, mycotoxin contamination and control strategies using ozone and chemical compounds. Doctoral Thesis Cranfield University.

Nesci, A., Gsponer, N., & Etcheverry, M. (2007). Natural Maize Phenolic Acids for Control of *Aflatoxigenic* Fungi on Maize. *Journal of Food Science*, *72*(5), M180–M185. https://doi.org/10.1111/j.1750-3841.2007.00394.x

Oyebanji, A. O., & Efiuvwevwere, B. J. O. (1999). Growth of spoilage mould and aflatoxin B1 production in naturally contaminated or artificially inoculated maize as influenced by moisture content under ambient tropical condition. *International Biodeterioration & Biodegradation*, *44*(4), 209–217. https://doi.org/10.1016/S0964-8305(99)00080-3

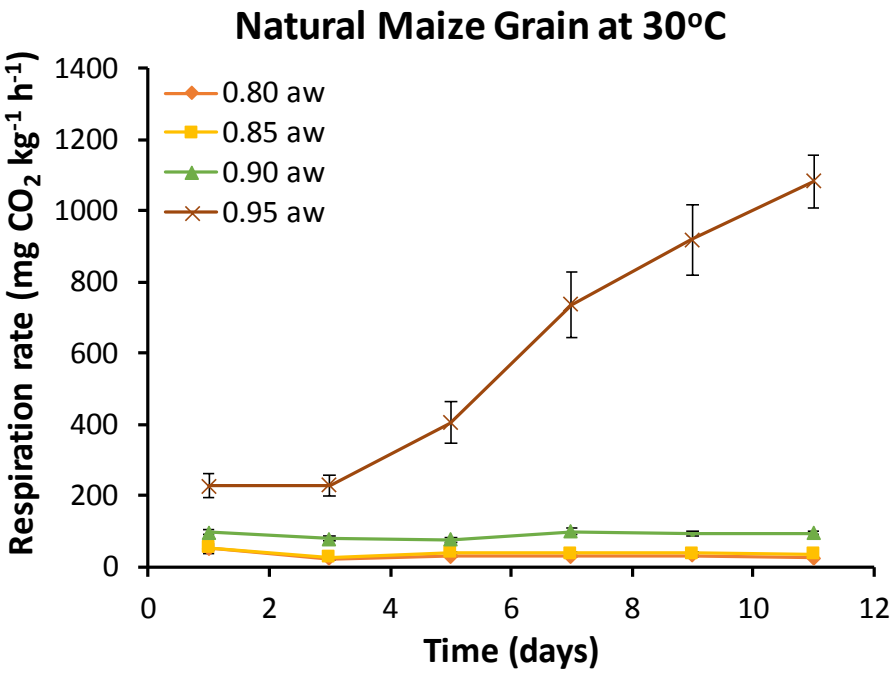
Picco, M., Nesci, A., Barros, G., Cavaglieri, L., & Etcheverry, M. (1999). Aflatoxin B1 and fumosin B1 in mixed cultures of *Aspergillus flavus* and *Fusarium proliferatum* on maize. *Natural Toxins*, *7*(6), 331–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11122525

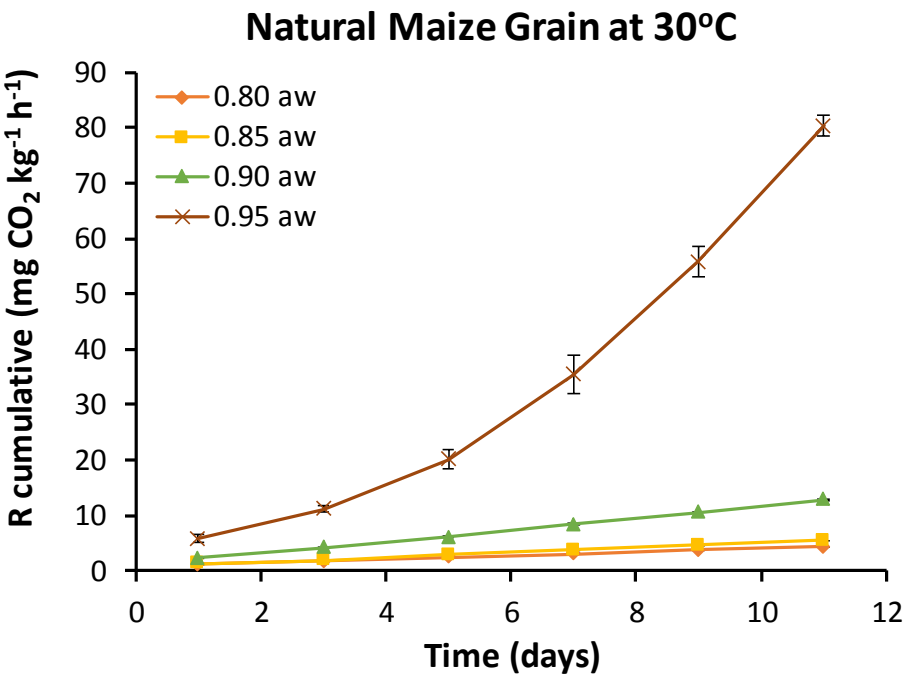
Samapundo, S., Devlieghere, F., Geeraerd, A. H., De Meulenaer, B., Van Impe, J. F., & Debevere, J. (2007). Modelling of the individual and combined effects of water activity and temperature on the radial growth of *Aspergillus flavus* and *A. parasiticus* on corn. *Food Microbiology*, *24*(5), 517–529. https://doi.org/10.1016/J.FM.2006.07.021

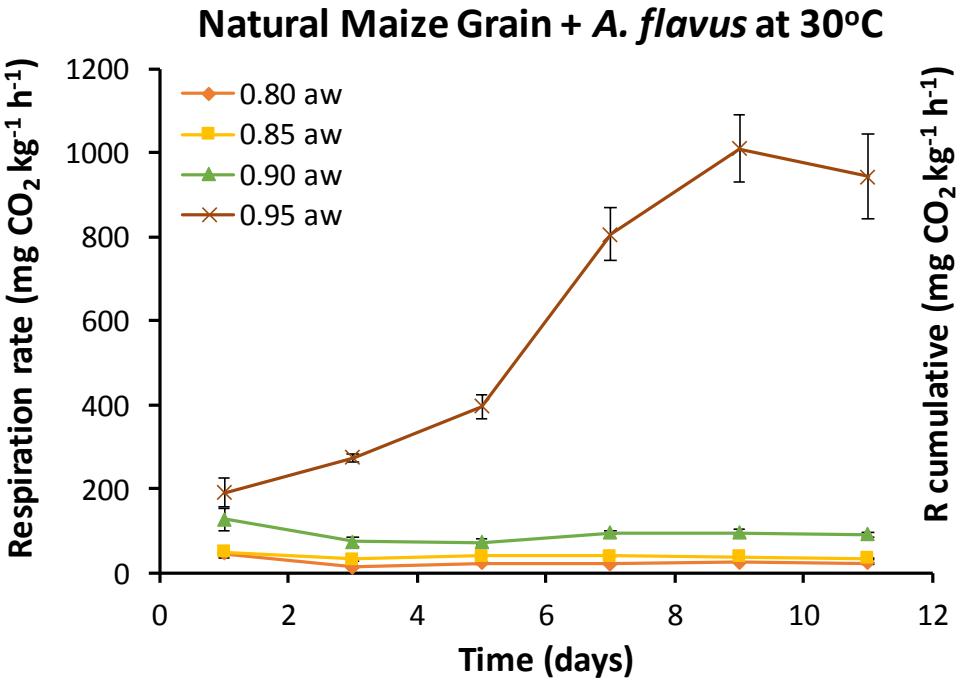
Saul, R. A., & Lind, E. F. (1958). Maximum Time for Safe Drying of Grain With Unheated Air. *Transactions of the ASAE*, *1*(1), 0029–0033. https://doi.org/10.13031/2013.41203

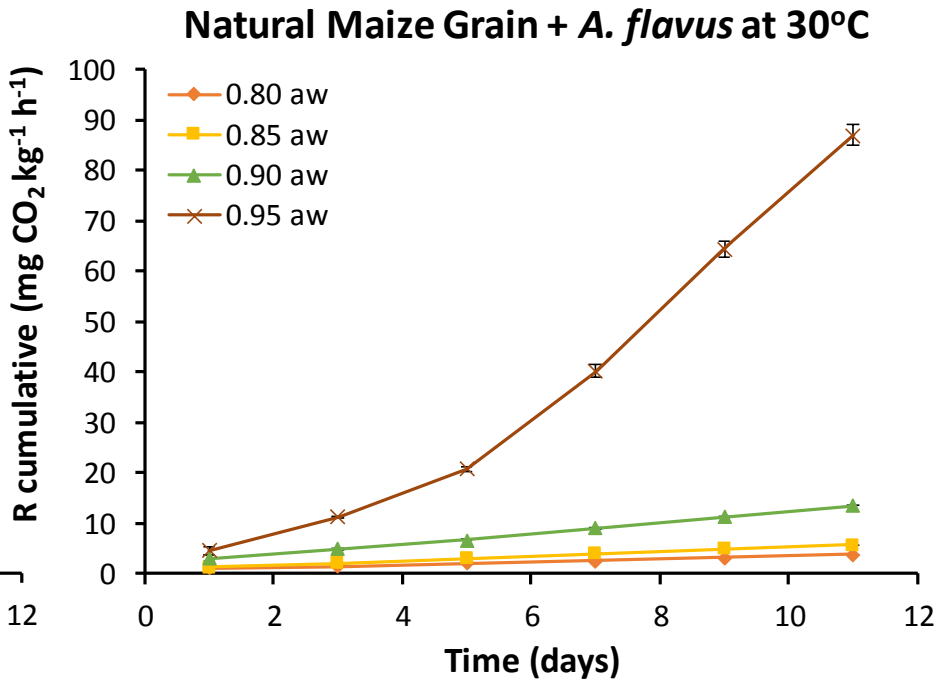
Seitz, L. M., Sauer, D. B., Mohr, H. E., & Aldis, D. F. (1982). Fungal growth and dry matter loss during bin storage of high-moisture corn. *Cereal Chemistry Journal*, *59*(1), 9–14.

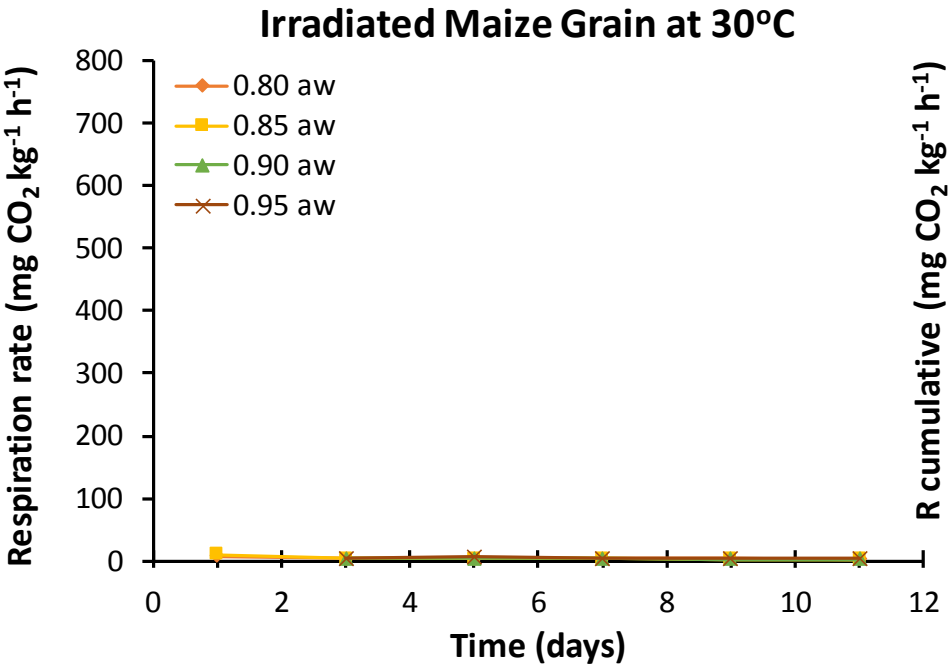
White, N. D. G., Sinha, R. N., & Muir, W. E. (1982). Intergranuar carbon dioxide as an indicator of biological activity associated with the spoilage of stored wheat., *V 24*(N 1), 35–42.

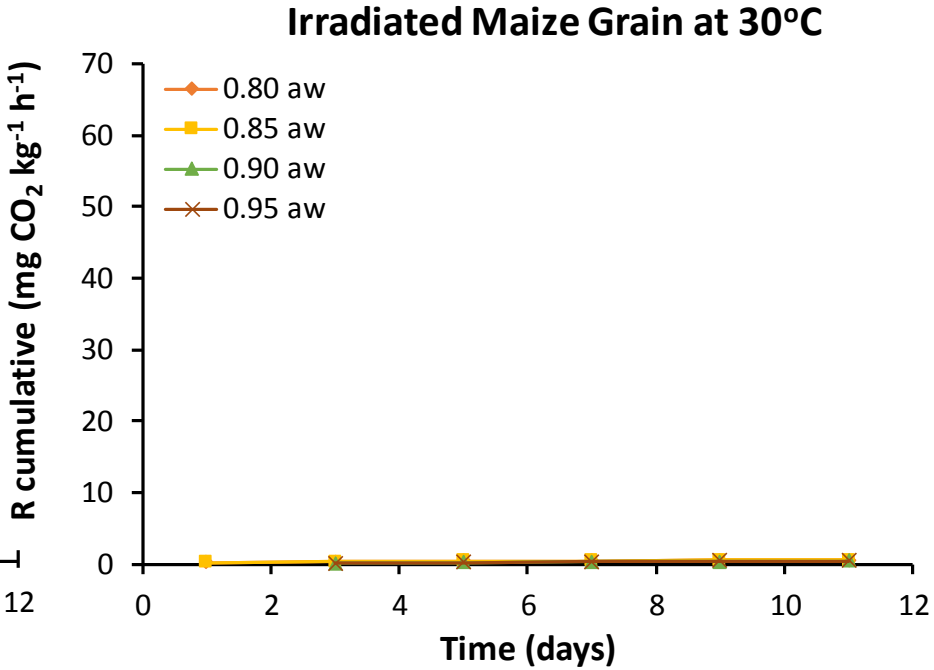

Natural Maize Grain at 30°C
Respiration rate (mg CO2 kg-1 h-1)
0.80 aw
0.85 aw
0.90 aw
0.95 aw
Time (days)

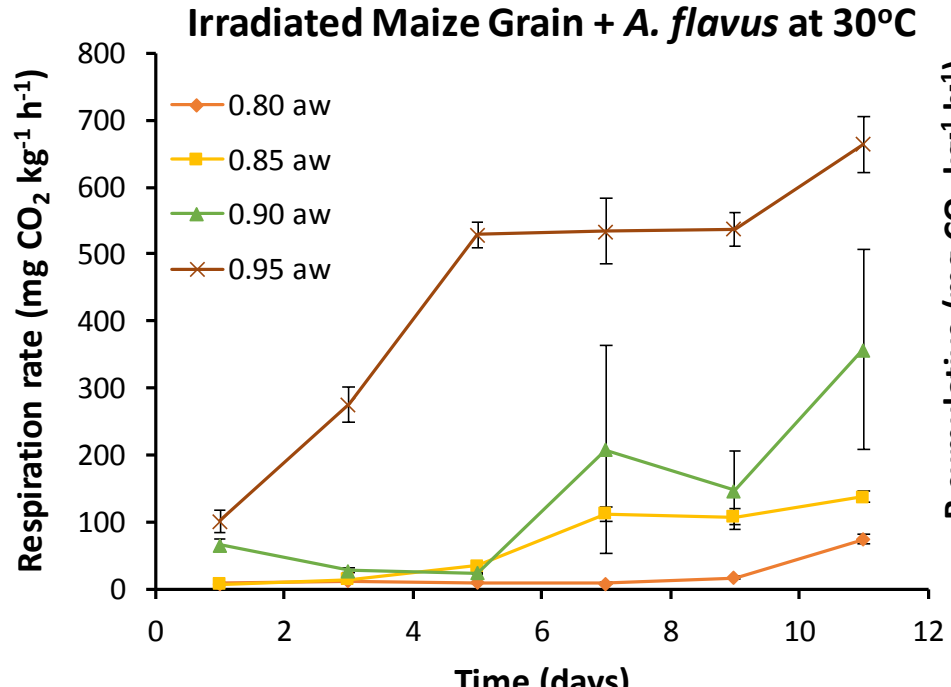

Natural Maize Grain at 30°C
R cumulative (mg CO2 kg-1 h-1)
0.80 aw
0.85 aw
0.90 aw
0.95 aw
Time (days)

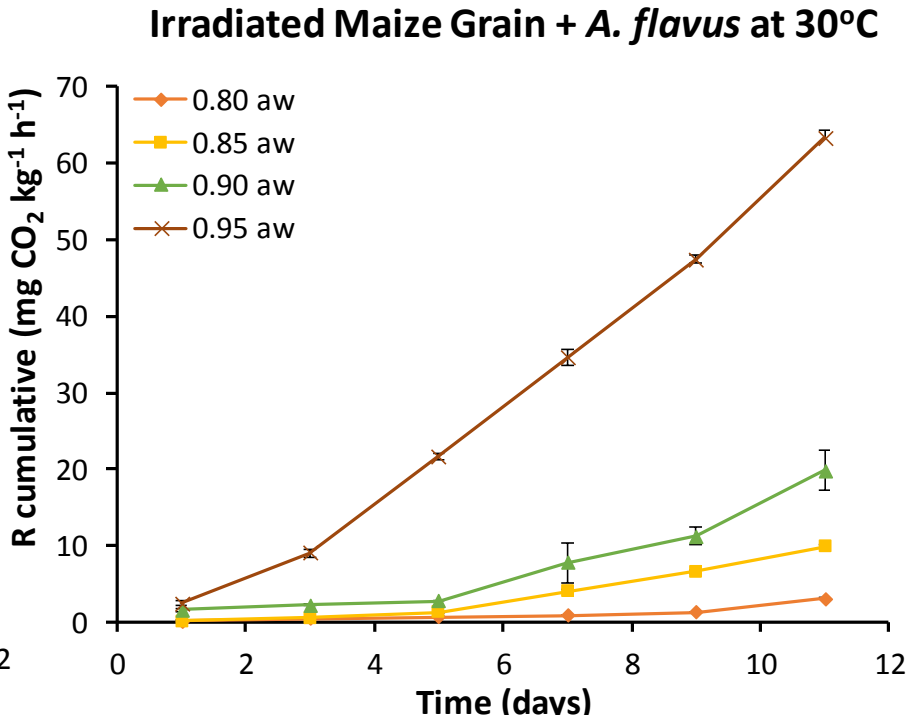

Natural Maize Grain + A. flavus at 30°C
Respiration rate (mg CO2 kg-1 h-1)
0.80 aw
0.85 aw
0.90 aw
0.95 aw
Time (days)


Natural Maize Grain + A. flavus at 30°C
R cumulative (mg CO2 kg-1 h-1)
0.80 aw
0.85 aw
0.90 aw
0.95 aw
Time (days)

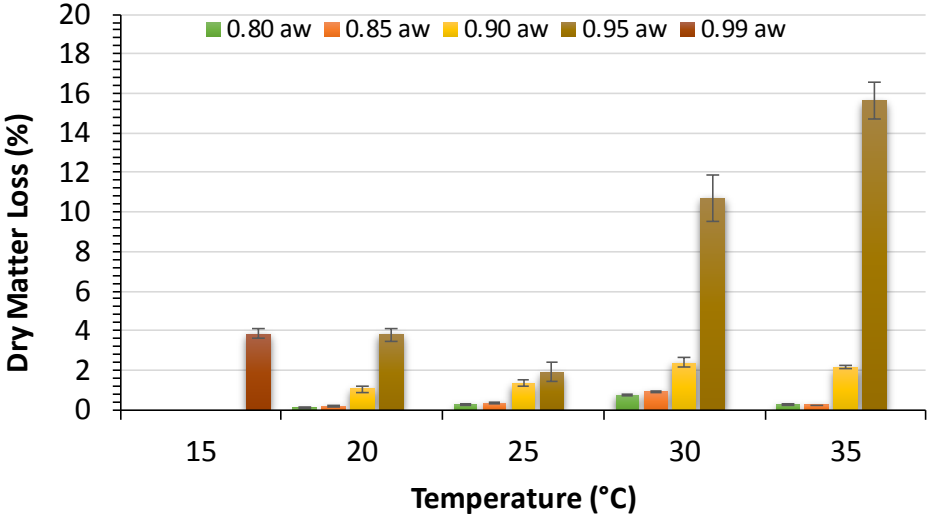

Irradiated Maize Grain at 30°C
Respiration rate (mg CO2 kg-1 h-1)
0.80 aw
0.85 aw
0.90 aw
0.95 aw
Time (days)


Irradiated Maize Grain at 30°C
R cumulative (mg CO2 kg-1 h-1)
0.80 aw
0.85 aw
0.90 aw
0.95 aw
Time (days)

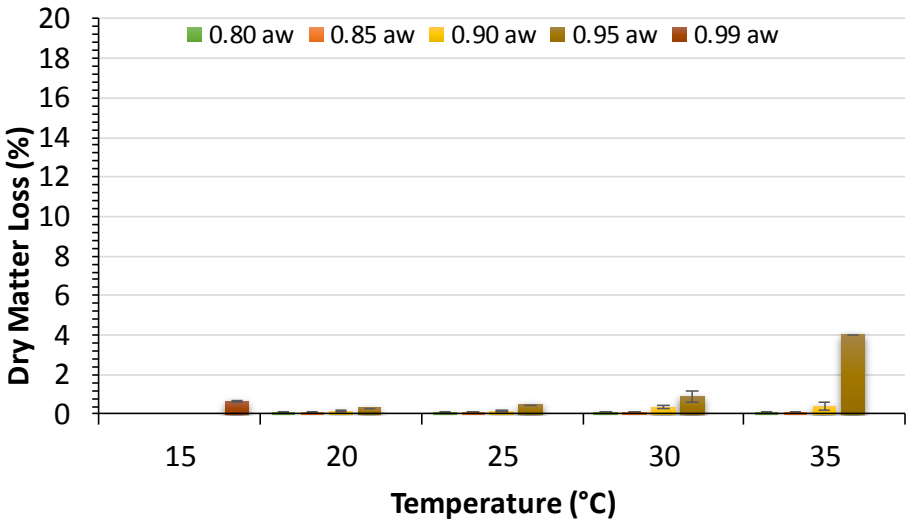

Irradiated Maize Grain + A. flavus at 30°C
Respiration rate (mg CO2 kg-1 h-1)
0.80 aw
0.85 aw
0.90 aw
0.95 aw
Time (days)


Irradiated Maize Grain + A. flavus at 30°C
R cumulative (mg CO2 kg-1 h-1)
0.80 aw
0.85 aw
0.90 aw
0.95 aw
Time (days)

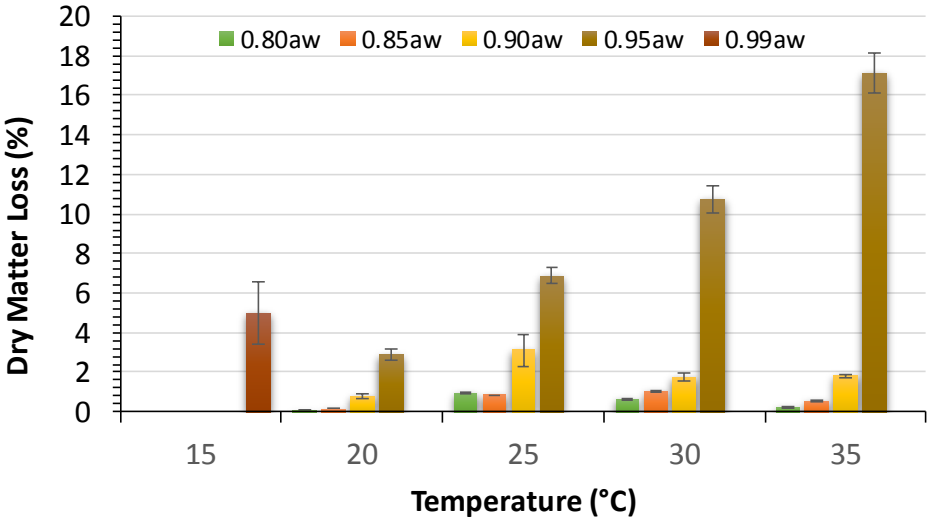
Natural Maize



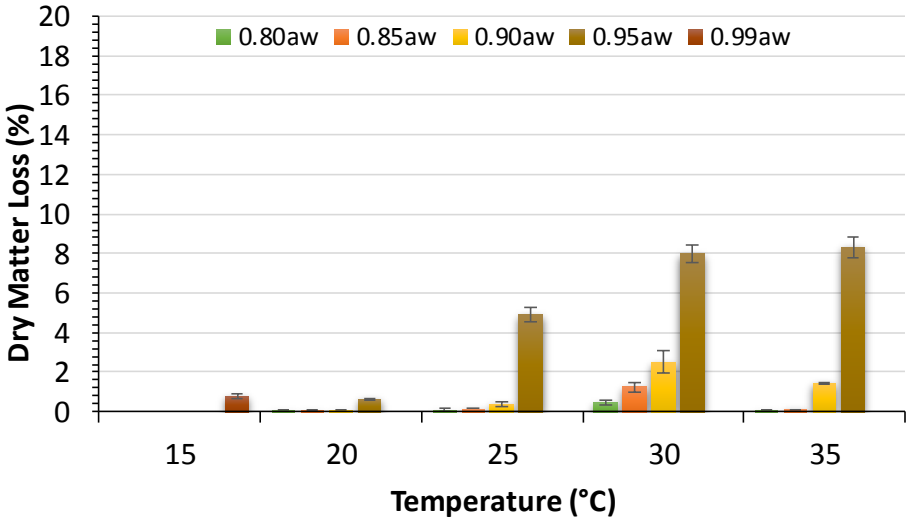
Irradiated Maize

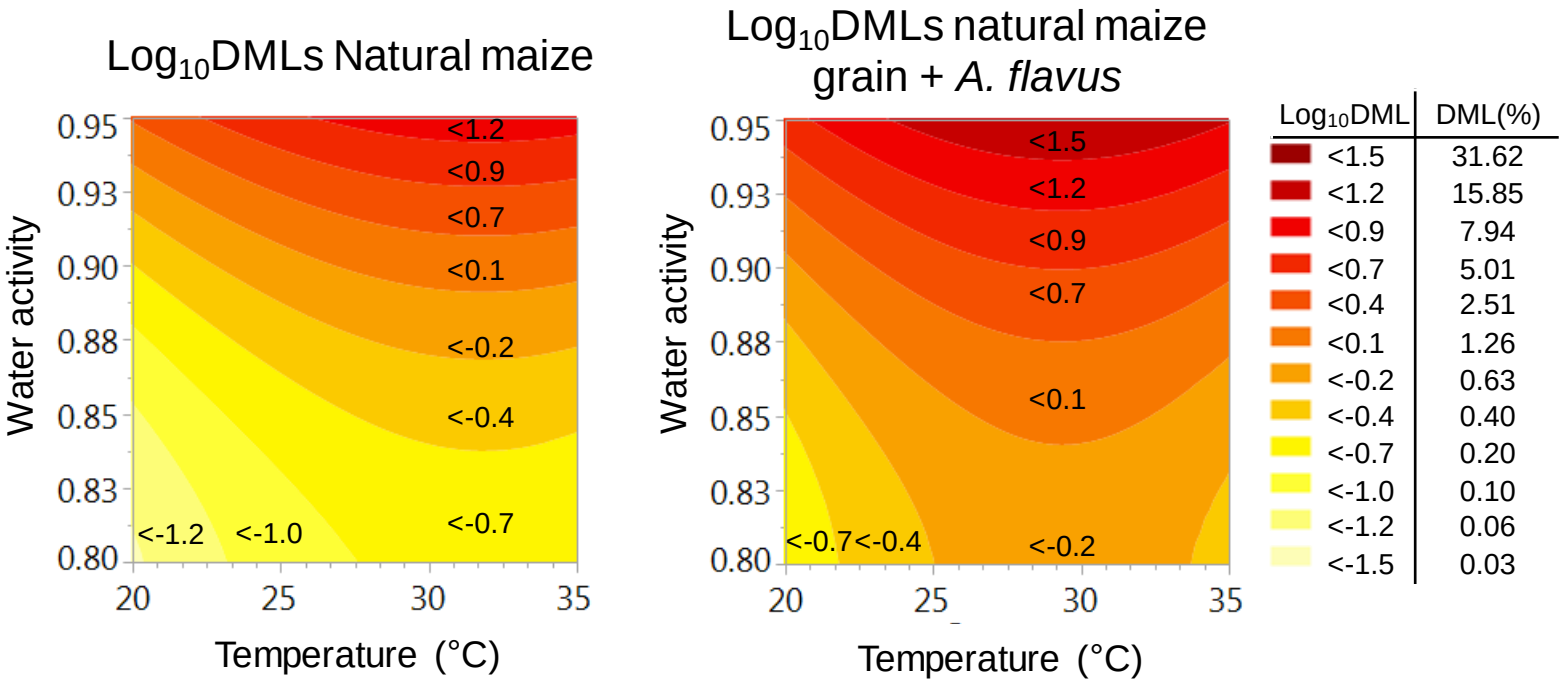


Natural Maize + *A. flavus*



Irradiated Maize + *A. flavus*




Log10DMLs Natural maize
Water activity
0.95
0.93
0.90
0.88
0.85
0.83
0.80
20
25
30
35
Temperature (°C)
<1.2
<0.9
<0.7
<0.1
<-0.2
<-0.4
<-0.7
<-1.2
<-1.0
Log10DMLs natural maize grain + A. flavus
<1.5
<1.2
<0.9
<0.7
<0.1
<-0.2
<-0.7<-0.4
Log10DML
DML(%)
<1.5 31.62
<1.2 15.85
<0.9 7.94
<0.7 5.01
<0.4 2.51
<0.1 1.26
<-0.2 0.63
<-0.4 0.40
<-0.7 0.20
<-1.0 0.10
<-1.2 0.06
<-1.5 0.03

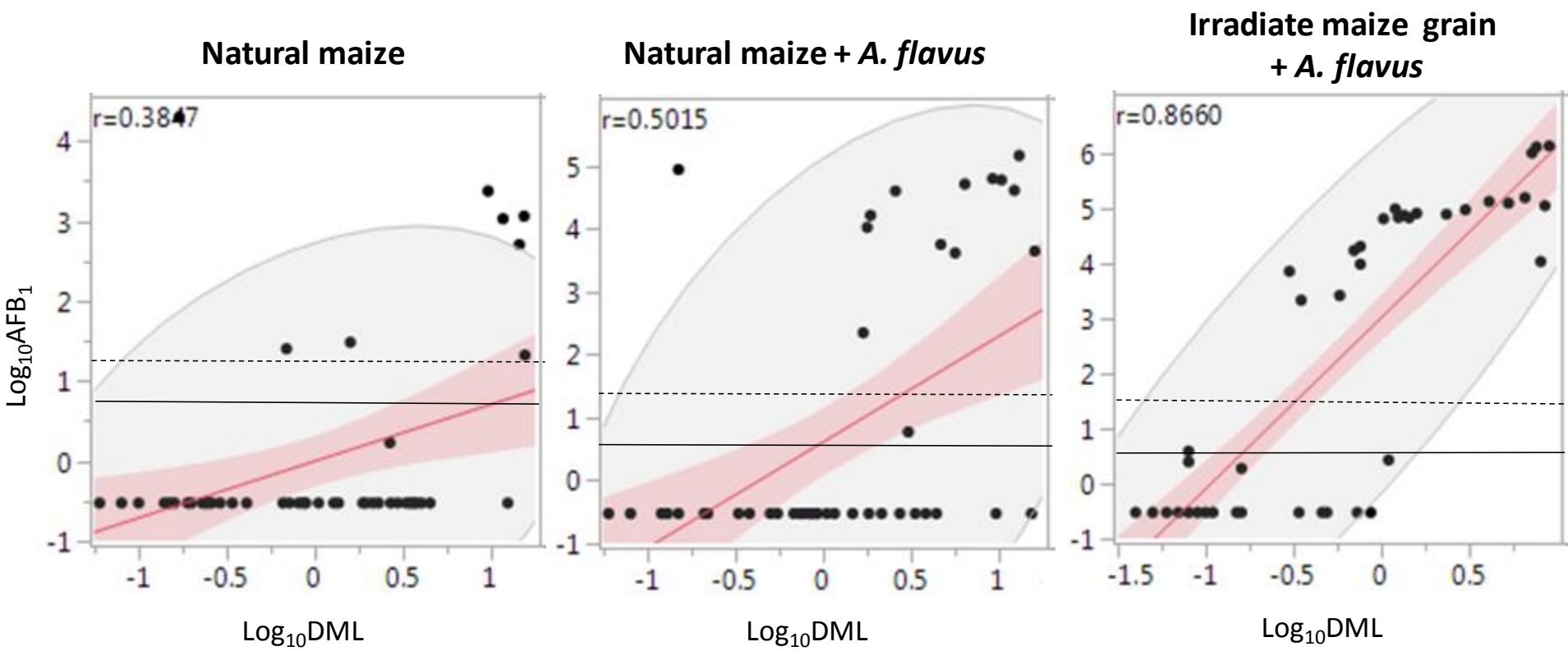

Natural maize
Natural maize + A. flavus
Irradiate maize grain + A. flavus
r=0.3847
r=0.5015
r=0.8660
Log10AFB1
Log10DML
Log10DML
Log10DML
Human consumption EU, 1881/2006 (5µg/kg)
Animal Feed EU, 2003/100 (20 µg/kg)

Table 1. Values of coefficients b_0-b_4, statistical significance of the relevant factor in the model equation for Log_{10}DLMs and toxin production as determined by forward stepwise regression.

	Log_{10}DMLs maize ± SD							
	Control				Inoculate			
Intercept (b_0)	-10.23	±	0.65	**	-10.05	±	0.51	**
Term (b1)	0.04	±	0.01	**	0.04	±	0.01	**
a_W (b_2)	10.29	±	0.67	**	10.45	±	0.53	**
T^2 (b3)	-0.004	±	0.001	*	-0.007	±	0.001	**
a_w^2 (b_4)	48.89	±	13.45	*	56.75	±	10.69	**
R^2 Adj	0.83				0.89			

***p-value*<0.0001 and **p-value*<0.005

Table 2. Aflatoxins contamination in different maize treatment under different environmental conditions after 11 days storage.

T(°C)	a_w	Natural maize			Natural maize + *A. flavus*			Irradiated grain + *A. flavus*		
		AFB_1	AFB_2	AFBs	AFB_1	AFB_2	AFBs	AFB_1	AFB_2	AFBs
15	0.99	0.3	0.3	0.6	0.3	0.3	0.6	1.1	0.3	1.4
20	0.8	0.3	0.3	0.6	0.3	0.3	0.6	0.3	0.3	0.6
20	0.85	0.3	0.3	0.6	0.3	0.3	0.6	0.3	0.3	0.6
20	0.9	0.3	0.3	0.6	0.3	0.3	0.6	1.5	0.3	1.8
20	0.95	0.3	0.3	0.6	76.2	4.2	80.3	13500	671	14100
25	0.8	0.3	0.3	0.6	0.3	0.3	0.6	0.3	0.3	0.6
25	0.85	0.3	0.3	0.6	0.3	0.3	0.6	0.8	0.3	1.1
25	0.9	10.4	0.8	11.1	0.3	0.3	0.6	6380	69.8	6440
25	0.95	9.1	0.3	9.4	20200	1290	21500	128000	3670	131600
30	0.8	0.3	0.3	0.6	0.3	0.3	0.6	1.0	0.3	1.3
30	0.85	0.3	0.3	0.6	0.3	0.3	0.6	67300	2880	70200
30	0.9	0.3	0.3	0.6	16700	524	17200	58000	2610	60600
30	0.95	1140	32.4	1180	88700	5730	94400	1240000	15600	1250000
35	0.8	6580	72.5	6650	28900	1000	29900	0.3	0.3	0.6
35	0.85	0.3	0.3	0.6	0.3	0.3	0.6	0.3	0.3	0.6
35	0.9	0.3	0.3	0.6	8110	257	8370	85300	2970	88300
35	0.95	559	15.1	574	14900	903	15800	93800	6830	1010000

AFB1 Maximum SE natural maize: 87719µg/kg: natural maize + *A. flavus:* 5370µg/kg and irradiate maize +*A. flavus:* 40819µg/kg
AFB2 Maximum SE natural maize: 2934µg/kg: natural maize + *A. flavus:* 102µg/kg and irradiate maize +*A. flavus:* 2832µg/kg
LOD/2 was considered when samples were <LOD.
Shading is per column, within columns the heat maps show that the red and then the amber are higher concentrations than the yellow treatments.

Supplementary table A. Statistical results p-values

	DML(%)							
	Natural maize grain		**Natural maize grain + *A. flavus***		**Irradiate maize grain**		**Irradiate maize grain + *A. flavus***	
	Level - Level	**p-Value**	**Level - Level**	**p-Value**	**Level - Level**	**p-Value**	**Level - Level**	**p-Value**
T	20-15	**<.0001**	20-15	**<.0001**	20-15	**<.0001**	20-15	**<.0001**
	25-15	**<.0001**	25-15	**0.0038**	25-15	**<.0001**	25-15	**<.0001**
	25-20	0.302	25-20	**<.0001**	25-20	0.741	25-20	**<.0001**
	30-15	0.0013	30-15	**0.0068**	30-15	**<.0001**	30-15	**<.0001**
	30-20	<.0001	30-20	**<.0001**	30-20	0.3494	30-20	0.0261
	30-25	<.0001	30-25	0.1289	30-25	0.4753	30-25	0.3892
	35-15	0.0008	35-15	0.0059	35-15	<.0001	35-15	0.1333
	35-20	0.0009	35-20	<.0001	35-20	0.2956	35-20	**0.0089**
	35-25	0.0264	35-25	0.9023	35-25	0.1459	35-25	**<.0001**
	35-30	**0.0004**	35-30	0.0308	35-30	0.0454	35-30	**<.0001**
a_W	0.85-0.80	0.0658	0.85-0.80	**0.0005**	0.85-0.80	0.7489	0.85-0.80	0.1049
	0.9-0.8	**<.0001**	0.9-0.8	**<.0001**	0.9-0.8	**<.0001**	0.9-0.8	**<.0001**
	0.9-0.85	**<.0001**	0.9-0.85	**<.0001**	0.9-0.85	**<.0001**	0.9-0.85	**<.0001**
	0.95-0.8	**<.0001**	0.95-0.8	**<.0001**	0.95-0.8	**<.0001**	0.95-0.8	**<.0001**
	0.95-0.85	**<.0001**	0.95-0.85	**<.0001**	0.95-0.85	**<.0001**	0.95-0.85	**<.0001**
	0.95-0.90	**<.0001**	0.95-0.90	**<.0001**	0.95-0.90	**0.0004**	0.95-0.90	**<.0001**
	0.99-0.8	**<.0001**	0.99-0.8	**<.0001**	0.99-0.8	**<.0001**	0.99-0.8	**<.0001**
	0.99-0.85	**<.0001**	0.99-0.85	**<.0001**	0.99-0.85	**<.0001**	0.99-0.85	**<.0001**
	0.99-0.9	**<.0001**	0.99-0.9	**0.0005**	0.99-0.9	**<.0001**	0.99-0.9	0.1145
	0.99-0.95	0.4226	0.99-0.95	**0.0011**	0.99-0.95	0.0677	0.99-0.95	**<.0001**

Nonparametric Comparisons for each pair using Wilcoxon Method
Grey numbers $p<0.05$. Bold numbers $p<0.01$

Supplementary table B. Statistical results p-values

		Natural maize grain		Natural maize grain + *A. flavus*		Irradiate maize grain + *A. flavus*	
		AFB_1	AFB_2	AFB_1	AFB_2	AFB_1	AFB_2
	Level - Level	**p-Value**	**p-Value**	**p-Value**	**p-Value**	**p-Value**	**p-Value**
a_W	0.85-0.80	0.3593	-	0.3593	0.3593	0.1326	0.0505
	0.9-0.8	1	-	0.7145	0.7145	**0.0008**	**0.001**
	0.9-0.85	0.3593	-	0.3066	0.3066	0.0311	0.1231
	0.95-0.8	0.0277	-	**0.0018**	**0.003**	**<.0001**	**<.0001**
	0.95-0.85	**0.0029**	-	**0.0001**	**0.0004**	**0.0005**	**0.0004**
	0.95-0.90	0.0133	-	0.0109	**0.0099**	0.0042	**0.0071**
	0.99-0.8	0.7389	-	0.7389	0.7389	0.2723	0.7389
	0.99-0.85	1	-	1	1	0.9315	0.4092
	0.99-0.9	0.7389	-	0.6433	0.6433	0.091	0.0802
	0.99-0.95	0.1175	-	0.0331	0.0526	0.0115	0.0112

Nonparametric Comparisons for each pair using Wilcoxon Method
Grey numbers $p<0.05$. Bold numbers $p<0.01$