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Comparison of dry matter losses and aflatoxin B_1 contamination of paddy and brown rice stored naturally or after inoculation with *Aspergillus flavus* at different environmental conditions

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ABSTRACT

The objective of this study was to compare the effect of different storage moisture conditions (0.70, 0.85, 0.90 and 0.95 water activity, a_w) and temperatures (20, 25, 30^{o}C) on (a) respiration rates (R) and dry matter loss (DML) of paddy and brown rice and (b) quantify aflatoxin B_1 (AFB_1) production by isolates of *Aspergillus flavus* from the rice samples and (c) inoculation of both rice types with *A. flavus* under these storage conditions on R, DML and AFB_1 contamination. There was an increase in temporal CO_2 production with wetter and warmer conditions in naturally contaminated rice. Higher R and consequently, % DML, were generally found in the brown rice (21%) while in paddy rice this was only up to 3.5% DML. From both rice types, 15 (83.3%) of 18 *A. flavus* isolates produced detectable levels of AFB_1 in a range 2.5-1979.6 µg/kg. There was an increase in DML in both rice types inoculated with *A. flavus* as temperature and a_w were increased. Interestingly very little AFB_1 was detected in paddy rice, but significant contamination occurred in the brown rice. The %DML in the control and *A. flavus* inoculated rice increased with temperature and a_w at both 25 and 30^{o}C from 1-2% to 15-20% DML at 30^{o}C and 0.95 a_w. All the inoculated rice samples had AFB_1 levels above the EU legislative limits for contamination in other temperate cereals and products derived from cereals (=2 µg/kg). Even samples with % DML as low as 0.2% had AFB_1 contamination levels twice the limits for other cereals. These results suggest that the mycotoxin contamination risk in staple commodities like rice, is influenced by whether the rice is processed or not, and that measurement of R rates can be used to predict the relative risk of AFB_1 contamination in such staple commodities.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important staple foods worldwide in conjunction with wheat and maize, with around 88% of consumption and 77% of exports concentrated in Asia (CGIAR Science Council, 2008). Therefore, the major rice-producing countries in 2016 were China, India and Indonesia, and according to the Food and Agriculture Organization of the United Nations (FAO), world rice production is steadily increasing, with a milled rice production of around 495.2 million tonnes (FAO, 2016).

As rice is often a staple component of the diet in many regions of the world it is essential to maintain high standards in terms of sensorial, nutritional and microbiological quality. The latter is highly affected by colonisation by some mycotoxigenic fungal species, in the genera *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* (Choi et al., 2015). Thus, mycotoxins constitute a major microbiological safety concern worldwide, especially in Asian countries because of the conducive warm and humid sub-tropical and tropical conditions which favours fungal growth and mycotoxin contamination of such commodities (Lai et al., 2015).

Currently, the presence of specific types of mycotoxins in rice, particularly aflatoxins (AFs) and more recently fumonisins (FUMs), is a subject of research interest. AFs are highly toxic, and are classed as Group 1a carcinogens (IARC, 2012). The most commonly found aflatoxin is AFB_1, followed by B_2, G_1 and G_2. While there are no legislative limits for total AFs/AFB_1 in rice, for other cereals there are strict EU legislative limits of 2 μg kg^{-1} for AFB_1 and 4 μg kg^{-1} for total AFs (Commission regulation (EC) no. 1881/2006). In other countries, the limits vary; e.g., China (20 μg kg^{-1}), India (30 μg kg^{-1}) and Malaysia (35 μg kg^{-1}; Liu et al., 2006). However, as rice is consumed in ever increasing amounts, potential contamination with AFs, even at low levels, could pose a risk to consumers (Paranagama et al., 2003).

Spoilage by mycotoxin-producing fungi can occur at different stages of cereal production; from the field to the warehouse, depending on a range of interacting abiotic and biotic factors (Magan and Aldred, 2007; Magan et al., 2010). In temperate and wet climates, *Fusarium* species are usually predominant as ''field flora'', while xerophilic fungi of the *Aspergillus* and *Penicillium* genera are prevalent during longer term storage (Fleurat-Lessard, 2002). Fungal colonisation under conducive environmental conditions can have a major effect in grain deterioration due to the progressive increase in the respiration rate (R), which consists of the oxidation of carbohydrates to produce carbon dioxide, water vapour and heat during aerobic respiration. Overall, a 1% dry matter loss (DML) corresponds to 14.7 g of CO_2 produced per kilogram cereal affecting the cereal quality (Kaleta and Górnicki, 2013). DML can be quantified on the basis of CO_2 production and respiration rates using gas chromatography (GC) (Magan et al., 2007; Mylona and Magan, 2011), and these data can be used as a ''storability risk index'' to predict overall quality changes in the grain. Previous studies by Mylona and Magan (2011) and Mylona et al. (2013) showed that only a small change in %DML (0.5-1.0%) caused by mycotoxigenic *Fusarium* species (*F. langsethiae*, *F. graminearum*, *F. verticillioides*) can lead to a significant increase in the relative risk of mycotoxin contamination, with toxin levels often above the EU legislative limits for type A and B trichothecenes and fumonisins in oats, wheat and maize respectively. However, very few studies have examined contamination of paddy and processed rice and impacts on mycotoxin contamination and quality Mousa et al., 2011; 2013).

The objectives of this study were to: (a) examine the effect of temperature x water activity (a_w) storage conditions on R of naturally contaminated paddy and brown rice, (b)

quantification of the R and total cumulative CO_2 production when these rice types were inoculated with *A. flavus*, (c) calculate and compare %DMLs due to natural fungal populations or that inoculated with *A. flavus* in paddy and brown rice, and (d) quantify the AFB_1 contamination levels in the paddy and brown rice and correlate AFB_1 and DMLs to determine the relationship between these two variables and the relative risk of exposure to AFB_1 in rice.

2. Materials and methods

2.1 Rice samples and development of the moisture adsorption curve

The initial a_w level of the paddy and brown rice from South East Asia were analysed using the AquaLAB Water Activity Meter 4 TE (Decagon Devices, Inc) at 25°C previously calibrated with a standard solution of 0.76 a_w. Known volumes of deionised water were added to 5-gram rice sub-samples in 25 ml Universal bottles, sealed and kept at 4°C for 48 hrs to allow equilibration, with regular shaking. The samples were then equilibrated at 25^{o}C and the a_w and the moisture content (m.c.; wet weight basis) determined. For m.c. determination samples were oven-dried at 125°C before weighing again. The results were then plotted to obtain a moisture adsorption isotherm for the two types of rice. The data for the amount of added water to achieve different a_w levels was also plotted to facilitate accurate modification of rice samples for storage experiments.

2.2 Fungal isolation and screening for aflatoxin B_1 production

Isolation procedure: Malt extract agar (MEA) and dichloran 18% glycerol media (DG18; both Oxoid Ltd., UK) were used to plate paddy and brown rice grains either directly (5 per Petri plate x 10 plates = 50 rice grains; for each medium) or after surface sterilisation using 1% sodium hypochlorite for 1 min followed by rinsing in sterile water for 30 secs and drying the rice grains on filter paper. The rice grains were placed equidistant on the MEA and DG18 agar media using a sterile forceps and incubated at 25°C for 10 days. Colonies of *Aspergillus* section *Flavi* were sub-cultured on MEA and DG18 for confirmation and for subsequent screening for potential production of aflatoxins. For comparison a type *Aspergillus flavus* strain (NRRL 3357; Northern Regional Research Laboratories (NRRL) of the U.S. Department of Agriculture USDA, New Orleans) was used.

Qualitative assay for aflatoxin production: The method used for aflatoxin screening with Coconut Agar Media (CAM) was adapted from Davis et al. (1987). For the media preparation, 250 mL of coconut cream (Sainsbury's, UK) were mixed with 250 mL of deionised water using a heated stirrer at 75°C. Once the solution was obtained, 10 g of Technical Agar No.3 (Oxoid Ltd., UK) and 0.08 g of chloramphenicol were added. The media was autoclaved and poured into 9 cm Petri dishes. After cooling, the plates were inoculated with a spore suspension of each *A. flavus* strain (18 strains) with a sterile loop. The inoculated Petri plates were incubated at 25°C for 7 days and further examined under UV light to check for presence/absence of a blue fluorescent ring, characteristic in aflatoxin-producing strains (Lin and Dianese, 1976). The aflatoxin-positive type strain (NRRL 3357) was used as a positive control.

Quantitative analysis using High Performance Liquid Chromatography (HPLC): The *A. flavus* isolates were subsequently cultured on a mycotoxin conducive Yeast Extract Sucrose agar

medium (YES, Oxoid Ltd., UK) for 10 days. After this time, 8 discs of each fungal culture (4 mm diameter, using a sterile cork-borer) were placed in previously weighed 2 mL volume safe-lock Eppendorf tubes. Aflatoxins were extracted with 1 mL of chloroform by shaking well for 1 h. The chloroform extract was transferred to a new vial and dried gently under air. Samples were derivatized using trifluoroacetic acid (TFA) as described in the AOAC method (2000). The samples were then transferred to sylanized HPLC vials using 1 mL syringes (Terumo Medical Corporation, UK) and filtering through 0.22 µm filters (Kromega, Jaytee Biosciences Ltd., UK). The HPLC equipment used was an Agilent 1200 Series system (Agilent, Berkshire, UK) with a fluorescence detector (FLD) (Millipore Waters, Corporation Massachusetts USA), excitation and emission wavelength of 360 and 440 nm, and flow rate of the mobile phase (methanol/water/acetonitrile, 30/60/10, v/v/v) of 1 ml min^{-1} for a running time of 25 min. Separation was achieved through the use of a C_{18} column (Poroshell 120 4.6 x 100 mm, 2.7 µm) preceded by a Phenomenex Gemini C_{18} 3 mm, 3 µm guard cartridge.

2.3 Respiration measurements by GC

Temporal respiration of naturally contaminated paddy and brown rice samples: Rice sub-samples (10 g) modified to different a_w levels with water (0.70, 0.80, 0.85, 0.90 and 0.95) and equilibrated as detailed previously were placed in 40-mL vials (Chromacol Ltd, UK) with sealable caps and a septum for gas removal. These were stored in 3 L chambers which also contained glycerol-water solutions to maintain the relative humidity (RH) of the atmosphere at the required ERH values at the different target temperatures (20, 25 and 30ºC). The sampling method used was based on that detailed previously by Mylona and Magan (2011) and 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 analysis was packed with Porapak Q and the data were analysed using Agilent Chemstation Software (Agilent Technologies, UK). The percentages of CO_2 concentration were used to calculate the R in mg CO_2 kg^{-1} h^{-1}, total cumulative production of CO_2 and the total DMLs (Dillahunty et al., 2000; Mylona and Magan, 2011).

Thus, the chromatograms obtained from the GC readings were used to calculate the CO_2 content of each sample using the following relationship:

$$\%\ CO2 = \left(\frac{10.2}{Area\ of\ the\ CO2\ standard}\right) \times Area\ of\ the\ sample$$

where 10.2 corresponds to the % CO_2 in the standard sample used for the daily calibration of the equipment. The values obtained were used to determine R, calculated as:

$$\frac{\left(\frac{\%\ CO2}{100}\right) \times V \times d}{m \times t}$$

where *V* is the volume of the headspace considering the added air during the sampling (45 ml in total); *d* is the density of CO_2 (1.977 mg/ml); and *m* and *t* are constant parameters that correspond to the dry weight of the sample (0.01 kg in this experiment) and the time period (1 hour), respectively.

Percentages of DMLs for each condition were calculated with the following formula:

$$\%DML = \frac{R \times T}{14.7\ \times 1000}$$

where, T (h) corresponds to the total storage time (240 hrs in this experiment).

2.4 Temporal respiration of samples inoculated with A. flavus

A spore suspension (100 µL per sample) from 10-day-old cultures of the high AFB_1-producing *A. flavus* strain was obtained by flooding the plate with sterile water (containing 0.01% tween 80) and agitating the surface with a sterile spatula. The concentration of the suspension was determined using a haemocytometer (Olympus BX40 microscope, Microoptical Co.; slide Marienfeld superior, Germany) and adjusted by dilution with sterile water to 10^4 spores mL^{-1} to achieve an overall concentration of 10^2 spores per gram of sample. Paddy and brown rice samples previously modified to the target a_w conditions (0.85, 0.90 and 0.95) were inoculated and stored at two temperatures (25 and 30°C). The respiration measurements were made as described previously. All experiments were fully replicated.

2.5 Aflatoxin B_1 analysis of colonised and stored rice samples

10-gram rice samples were oven-dried at 60°C for 48 hours and then milled in a laboratory blender (Waring Commercial, Christison, UK) for extraction and analysis for quantification with HPLC-FLD. To this end AFB_1 extraction was performed using the AflaStar™R - Immunoaffinity Columns (IAC, Romer Labs Inc., MO, USA) in 5 gram sub-samples. Natural paddy and brown rice grain was also examined to obtain information on any background level of AFB_1 which might be present in the rice samples used.

2.6 Statistical Analysis

Statistical analysis was performed using the package JMP Pro 12 (SAS Institute, INC., Cary, NC, USA). Datasets were tested for homoscedasticity and normality using the Levene and Shapiro-Wilk-test, respectively. When data failed the normality test, variable transformation was performed to try to improve normality or homogenise the variances. Two-factor ANOVA was then used to find significant differences between groups. When comparing between two levels t-test was used. Comparisons were considered significantly different when p-values<0.05.

3. Results

3.1 Evaluation of the ability of A. flavus *strains to produce aflatoxins*

The results in CAM showed the strains were producing a yellowish pigmentation that then led to the blue fluorescence under UV light as previously described by Lin & Dianese (1976). This was linked to aflatoxin-producing isolates. This colouration was not detected in non-producing colonies. However, the blue fluorescence did not appear in all the potentially mycotoxigenic isolates. Based on these observations all isolated strains were subsequently analysed using HPLC to compare the obtained results.

Further HPLC-FLD analysis of AFs showed that 15 (83.3 %) of 18 tested *A. flavus* isolates produced detectable levels of AFB_1 at concentrations ranging from 2.5 to 1979.6 µg kg^{-1}. None of the isolates produced AFB_2, AFG_1 or AFG_2. This suggested that they were all *A. flavus* strains. The most potent producer (BRAF10) was used to inoculate the rice samples in the subsequent experiments.

3.2 Influence of water activity and temperature on the temporal respiration rate and total accumulated CO_2 production in stored paddy and brown rice

Hourly respiration rates from both types of rice were used to calculate the total accumulated CO_2 produced during colonisation by the natural mycobiota (in g kg^{-1}) over the storage period. Figure 1a shows the temporal production of CO_2 in paddy rice, and Figure 1b the total accumulated CO_2 production over 10 days at 25°C at the three a_w levels examined. This showed that optimum production was at 0.95 a_w followed by the other a_w levels of the paddy rice. Figure 2a and 2b show the results under the same conditions for brown rice. Both the temporal and accumulated CO_2 production was much higher in this type of rice than the paddy rice although the latter, in terms of maximum rates followed a similar pattern, regardless of a_w. The rate of CO_2 production and the total R amounts were higher at 30°C, although the patterns were the same (data not shown). The highest R in both types of rice was found at 0.95 a_w and increased with time and temperature.

When the paddy and brown rice were inoculated with *A. flavus*, respiration was constantly very low at 0.85 and 0.90 a_w, but it increased slowly with time over the 10-day storage period (Figure 3a,b; Figure 4a,b). The R rate in brown rice was 3-4x greater than that in paddy rice.

3.3 Relationship between storage environmental conditions and dry matter losses (DMLs) in naturally stored paddy and brown rice and that inoculated with A.flavus*.*

Figure 5 and 6 compare the relative DMLs of naturally contaminated paddy and brown rice and that inoculated with *A. flavus* after a 10-day storage period. The maximum DML in paddy rice was about 4% while in the brown rice this was almost 15-20% under the wettest conditions tested (0.95 a_w). Inoculation with *A. flavus* resulted in slightly increased DML, especially at 0.85 and 0.90 a_w, in brown rice. Two-factor ANOVA analysis showed that there was a significant effect of a_w on %DMLs in all the treatments ($p=0.05$).

3.4 Aflatoxin B_1 contamination of paddy and brown rice and correlation between DMLs and toxin contamination

The highest amounts of AFB_1 were found in both types of rice at 0.95 a_w + 30°C (maximum value: 14401.23 µg kg^{-1})(Figure 7). The lowest contamination level was at 0.85 a_w and 25°C. In all cases the paddy rice had significantly lower contamination levels after storage for 10 days, regardless of a_w and temperature. As the rice was found to have some low levels of AFB_1 contamination the amounts present were adjusted to take this into account. Statistical analyses showed a highly significant effect of a_w, temperature and a_w x temperature treatment effects ($p<0.05$) on AFB_1 production.

Plotting the data for AFB_1 against DMLs showed a highly significant positive correlation in the stored rice ($R = 0.9029$, $p < 0.01$). All the samples exceeded the EU legislative limits for AFB_1 in other temperate cereals and products derived from cereals, including processed cereal products (2 µg kg^{-1}). Even samples with %DMLs as low as 0.2% showed contamination levels twice this limit (Figure 8).

4. Discussion

The paddy and brown rice used in this study had a natural mycobiota which consisted of *Aspergillus*, *Alternaria*, *Penicillium*, *Rhizopus*, *Fusarium* and *Phoma*, among others. These were similar to the predominant fungal genera found previously on rice from different parts of the world (Reddy et al., 2009; Khosravi et al., 2013). The *A. flavus* isolates were predominantly able to produce AFB_1 both qualitatively and quantitative (±80%). However, it was important to carry out quantitative analyses as the CAM media was not able to identify strains which were very low producers of AFs (Lin & Dianese, 1976). Though, the appearance of a yellow pigmentation in the CAM media appeared to be a rapid and simple method to use as a qualitative test for AF-producing *A. flavus* strains. Nevertheless, to identify the high AFB_1 producer strains, quantification was done with HPLC-FLD.

Regardless of fungal contamination in paddy rice, the presence of the husk as a protective coating, had a significant effect on R, which was much lower than in the case of dehusked brown rice where fungi could easily utilise the starch in rice grains (Reddy et al., 2008). Simultaneously, the high nutrient availability in brown rice lead to more rapid fungal colonisation with much higher R and DMLs, especially in the wettest and warmest conditions tested (0.95 a_w and 30^{o}C). For paddy rice, the maximum DMLs were observed at 0.95 a_w + 25^{o}C rather than at 30^{o}C. This may be partially due to the fungal species naturally present having optimum growth conditions in these treatment conditions.

As found previously for oats and maize (Mylona and Magan, 2011; Mylona et al., 2012), the DMLs obtained were significantly influenced by a_w. The effect of temperature was not as significant in the present study. However, there was a statistically significant effect of a_w x temperature in paddy rice on DMLs. This may be due to the importance of the most favourable combination of environmental conditions for the colonisation of the paddy rice where it is probably more difficult to access the nutrients.

Addition of *A. flavus* inoculum had some effect on the colonisation rate of both rice types. The R and % DML appeared to be different where the natural mycobiota was enriched with spores of this mycotoxigenic species. The optimum conditions were slightly different based on cumulative R rates. DMLs at 30^{o}C was greater at 30^{o}C and 0.98 a_w when *A. flavus* inoculation was used compared to the un-inoculated control in paddy rice. In contrast, in brown rice where access to nutrients was better the in paddy rice, *A. flavus* inoculated rice had higher levels of DML, at 30 than 25^{o}C, regardless of a_w level tested.

AFB_1 contamination in both types of rice increased in the wettest conditions and highest temperatures. This is similar to what has been previously found for the optimal range for AFB_1 production being between 25-30ºC in paddy rice (Mousa et al., 2013) with a_w levels close to 0.99 (Mousa et al., 2011). The same authors also found a higher mycotoxin production at lower a_w levels (0.90; 0.92) at 25°C. This suggests that certain environmental stress conditions enhanced this production, as in the case of AFB_1 (Schmidt-Heydt et al., 2012). In a previous study with maize grain, the effect of a_w on AFB_1 contamination levels was more significant than temperature (Faraj et al., 1991). However, there are several studies that show differences regarding the optimal and minimum values of AFB_1 under interacting conditions of a_w x temperature due to differences in the range of conditions, the type of media and fungal strains studied. Mycotoxin contamination levels were again higher in brown than in paddy rice, confirming the physical protection exerted by the rice husk.

The high correlation found between AFB_1 accumulation in stored rice and DMLs suggests that very small changes in DML will influence rice quality. This study has shown

that 1-1.5% DML and values as low as 0.2% DML can lead to AFB_1 contamination, respectively. Previous studies by Mylona and Magan (2011) showed that <1% DML could lead to T-2 and HT-2 toxin contamination exceeding EU recommended limits in oats. For maize, Mylona et al. (2012) found that between 0.8-1.0% DML in maize due to *F. verticilliodies* colonisation can lead to FB_1 levels exceeding the EU limits. In the present study even with 0.2% DMLs due to *A. flavus* colonisation, the amount of AFB_1 exceeded the limits set for other cereals. Previously, the degree of milling of rice appeared to influence AFs contamination, with higher values in brown dehusked rice than in paddy rice (Choi et al., 2015). This does raise safety concerns for rice and emphasizes the possible need for examination the relative hazardous nature of such contamination and whether it represents a significant risk in stored rice, especially processed rice. In contrast, the limited ability to colonise paddy rice suggests that storage for the short and medium term may be better in this form than in a processed form.

These results also suggest that R and CO_2 production can be used to predict the risk of mycotoxin contamination. Innovative post-harvest management strategies including real time monitoring of rice respiration should perhaps be considered as an early indicator of fungal activity to prevent fungal colonisation and toxin contamination in polished rice. If this is combined with biological models of the boundary conditions for growth/no growth or mycotoxin/no mycotoxin production and linked to CO_2 production and DMLs then this could prove to be a useful tool for better post-harvest management in this important staple food chain.

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Figures

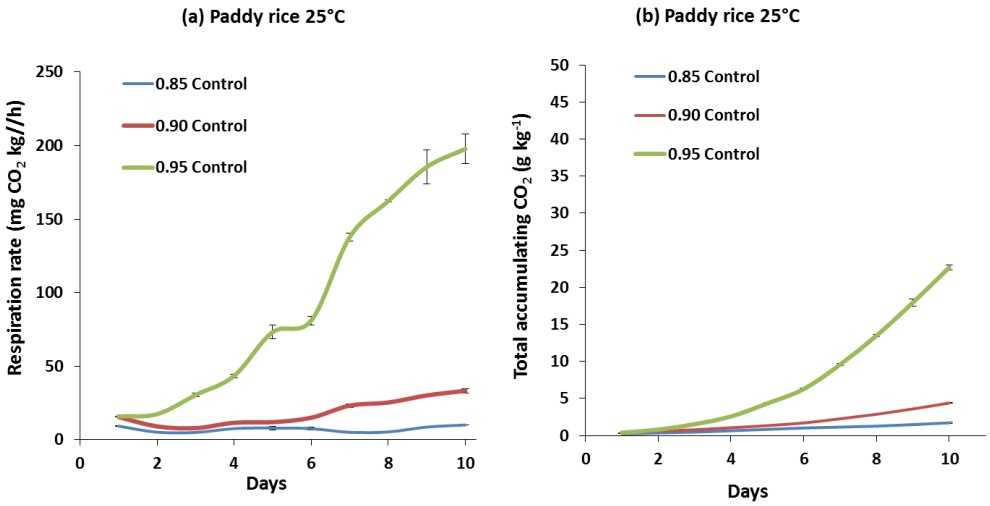

Figure 1. Martin et al.

Figure 1. (a) Temporal respiration and (b) total accumulation of CO_2 by naturally contaminated paddy rice stored at 25^{o}C for up to 10 days at three different water activity levels (a_w). Vertical bars represent standard errors of the mean.

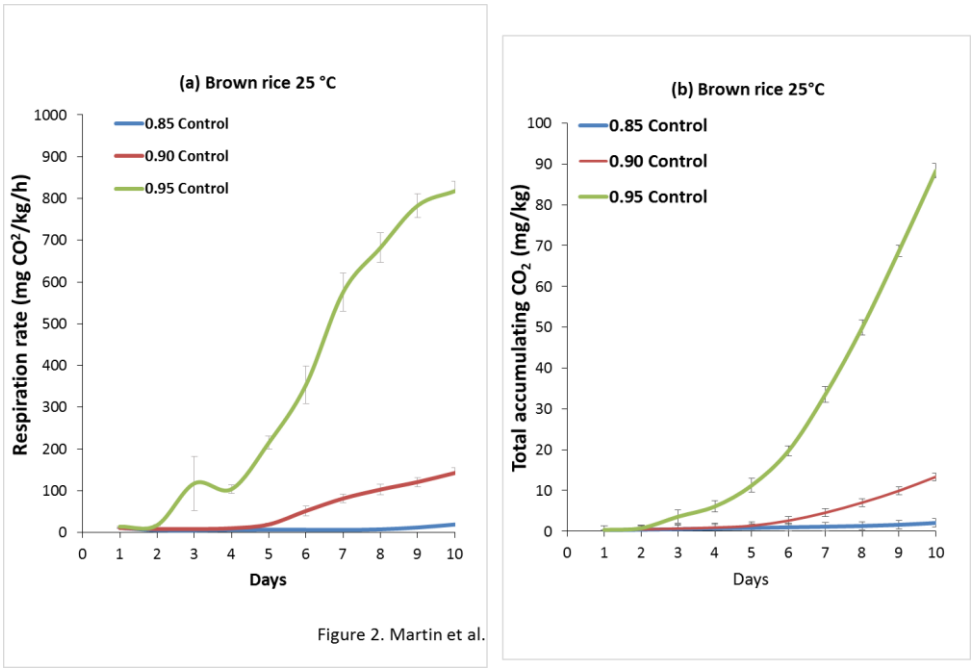

Figure 2. (a) Temporal respiration and (b) total accumulation of CO_2 by naturally contaminated brown rice stored at 25°C for up to 10 days at three different water activity levels (a_w). Vertical bars represent standard errors of the mean.

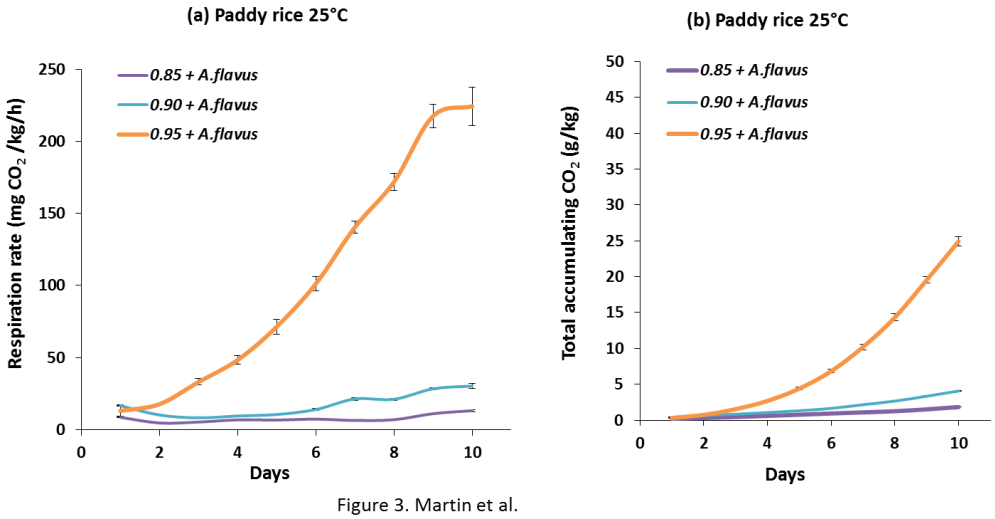

Figure 3. (a) Temporal respiration and (b) total accumulation of CO_2 by paddy rice inoculated with *Aspergillus flavus* and stored at 25°C for 10 days at three different water activity levels (a_w). Vertical bars represent standard errors of the mean.

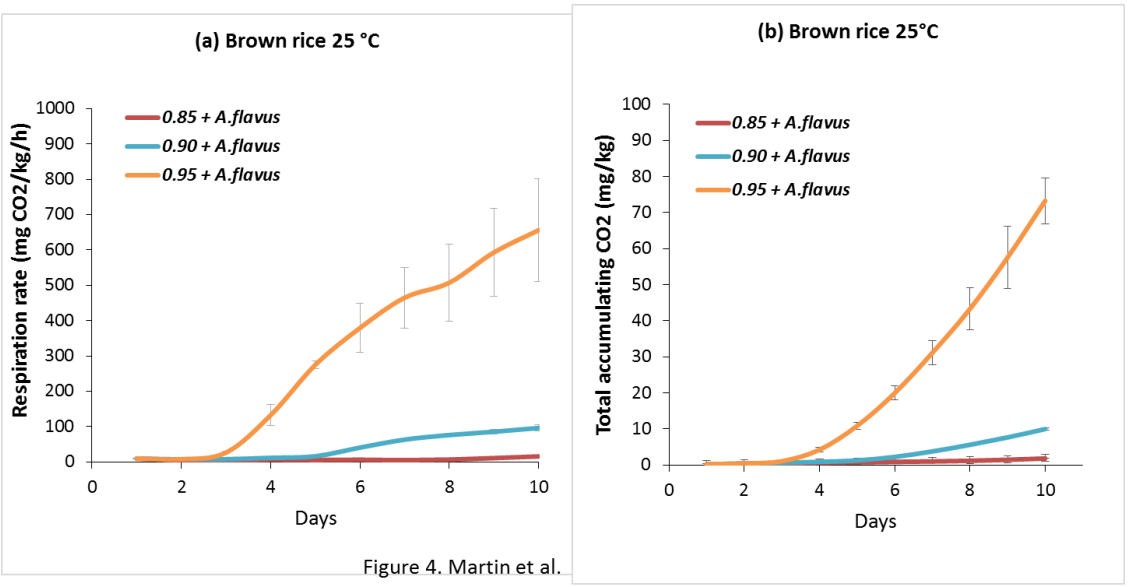

Figure 4. Martin et al.

Figure 4. (a) Temporal respiration and (b) total accumulation of CO_2 by brown rice inoculated with *Aspergillus flavus* and stored at 25°C for 10 days at three different water activity levels (a_w). Vertical bars represent standard errors of the mean.

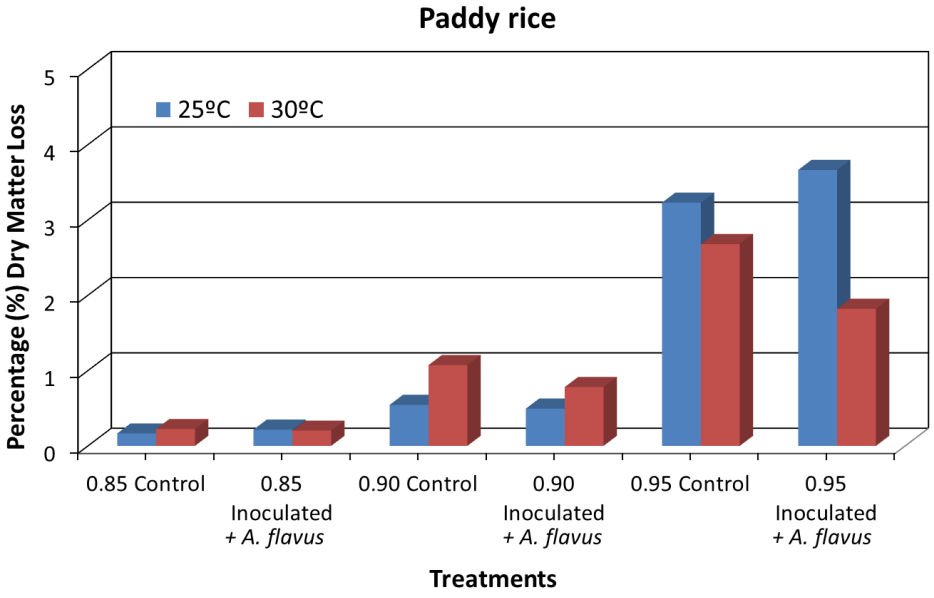

Figure 5. Martin et al.

Figure 5. Percentage of Dry Matter Loss (DML) in paddy rice due to natural spoilage or that inoculated with *A. flavus* at different a_w x temperature conditions after 10 days storage.

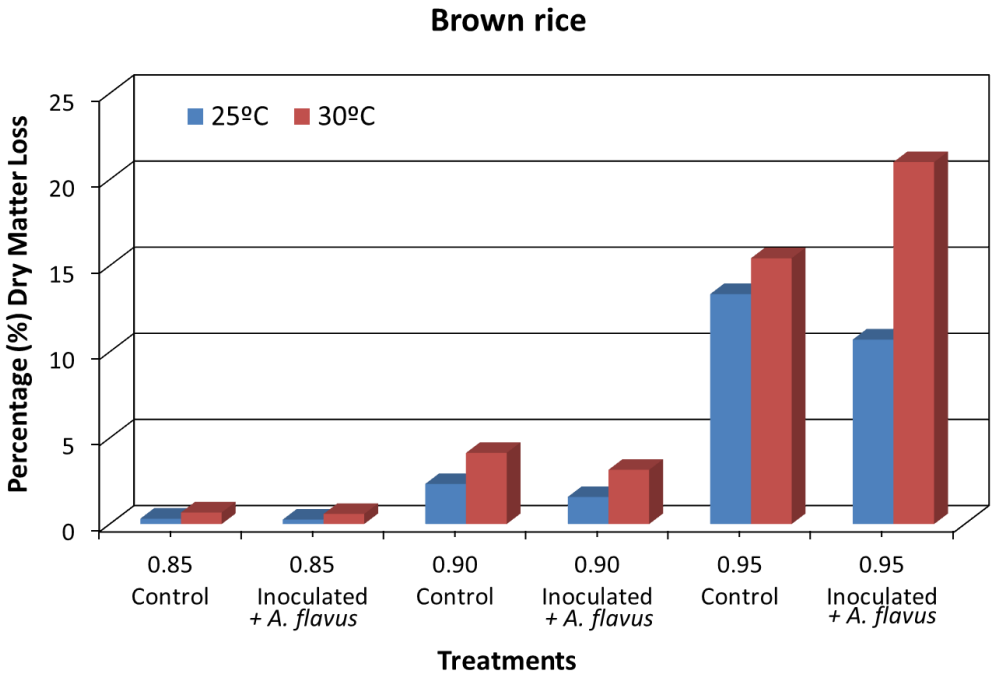

Figure 6. Martin et al.

Figure 6. Percentage of Dry Matter Loss (DML) in brown rice due to natural spoilage fungi or that inoculated with *A. flavus* at different a_w x temperature conditions after 10 days storage.

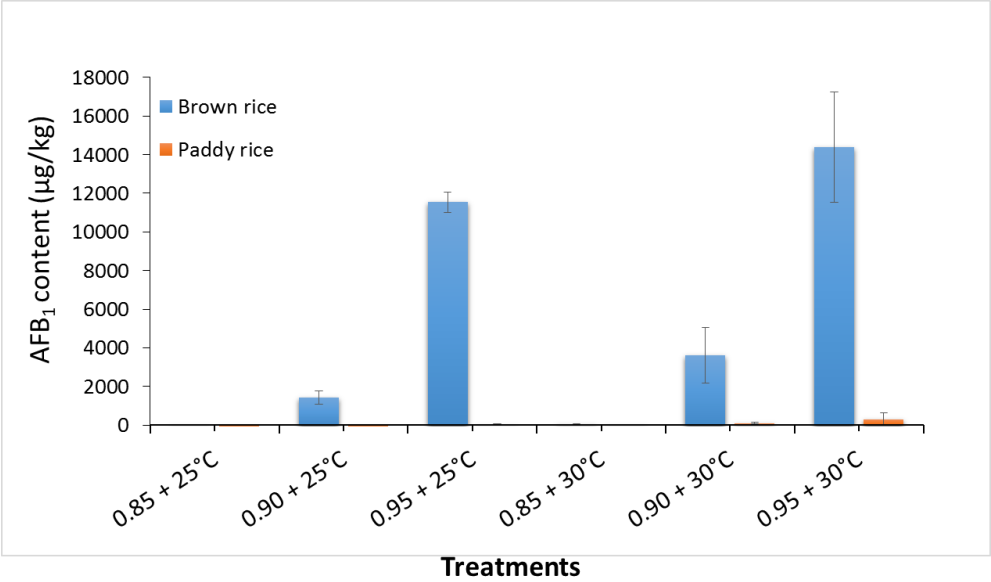

Figure 7. Martin et al.

Figure 7. Effect of a_w x temperature conditions on aflatoxin B_1 contamination of stored paddy and brown rice after 10-days storage. Vertical bars represent standard errors of the means (n=3).

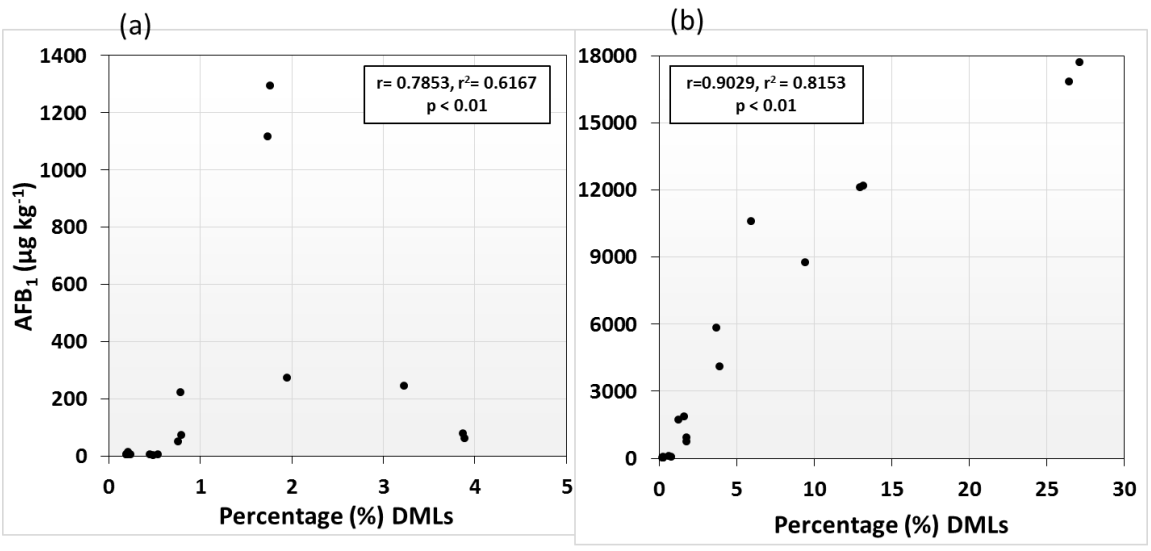

Figure 8. Martin et al.

Figure 8. Scatter plot of Dry Matter Loss (DMLs) and aflatoxin B_1 contamination of (a) paddy and (b) brown rice after 10 days storage under all the environmental conditions examined.

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

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http://dx.doi.org/10.1016/j.jspr.2017.06.004