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IN VITRO PRODUCTION OF TRICHOTHECENES AND ZEARALENONE BY *FUSARIUM* ISOLATES FROM EQUATORIAL BARLEY (*HORDEUM VULGARE* L.) GROWN IN KENYA

Mbae KM[1*], Kiiyukia C[2] and GM Kenji[3]

Kenneth Mbae

*Corresponding author email: muneneken@gmail.com

[1]Department of Food Science, Meru University College of Science and Technology P.O.BOX 972, Meru 60200, Kenya

[2]Institute of Tropical Medicine and Infectious Diseases, Jomo Kenyatta University of Agriculture and Technology P.O.BOX 62000 Nairobi 02000, Kenya

[3]Department of Food Science and Technology, Jomo Kenyatta University of Agriculture and Technology P.O.BOX 62000 Nairobi 02000, Kenya

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ABSTRACT

Fusarium head blight (scab) is a devastating disease of wheat and barley throughout the world. The disease has been reported worldwide wherever cereals are grown, cutting across diverse ecological and geographical distribution. In addition to being pathogenic to plants, which may cause severe crop yield reduction, many *Fusarium* species are also capable of producing mycotoxins deleterious to human health as secondary metabolites. *Fusarium* toxins are commonly detected in wheat, barley, maize, rice and beer. Traditionally malted barley (*Hordeum vulgare* L.) is the principal ingredient in clear beer and *Fusarium* toxins incidences are of major concern. Moreover, the spent grain from the brewing industry is used as feed and presence of mycotoxins can lead to harmful effects on domestic animals and also find a way into the human food chain. Studies carried out in Kenya have revealed presence of various *Fusarium* species with ability to produce mycotoxins and presence of *Fusarium* toxins in wheat and maize and beer. Based on the ubiquitous nature of *Fusarium* mold and the fact that barley production takes place in maize and wheat growing areas, this study set out to investigate the occurrence of *Fusarium* molds in Equatorial barley grown in Kenya and the ability of the isolates to produce selected mycotoxins. Grain samples were obtained from newly delivered barley lots originating from two regions and stored grain awaiting malting after break of dormancy from Kenya Maltings Ltd., Nairobi. The *Fusarium* isolates were identified to species level based on cultural and morphological characteristics. Additionally, they were screened *in-vitro* on rice cultures for their ability to produce Type A trichothecenes (T–2 toxin, HT–2 toxin, Diacetoxyscirpenol), Type B trichothecenes (deoxynivalenol and nivalenol) and Zearalenone. Samples from all sources were contaminated with *Fusarium*, but at varying magnitudes - 50%, 33.3% and 25% for barley kernels originating from Timau, Olchoro and in-storage grain with no common history of origin, respectively. The distribution of the species showed some regional specificity. *F. graminearum* and *F. poae* predominated in kernels sourced from Olchoro region. All strains of *F. graminearum* produced both deoxynivalenol and zearalenone. *F. poae* strains *and F. chlamydosporum* did not produce detectable amounts of the screened mycotoxins. However, two inconclusively identified isolates of *Fusarium spp*. isolated from Timau samples produced deoxynivalenol only. The study revealed that a number of toxigenic *Fusarium spp.* do occur in Equatorial barley grown in Kenya.

Key words: Barley, *Fusarium*, trichothecenes, zearalenone, Kenya.

INTRODUCTION

Most genera of cultivated plants are susceptible to diseases caused by *Fusarium* species including all members of the *Gramineae* family [1]. *Fusarium* head blight (scab) is a devastating disease of wheat and barley throughout the world [2]. The disease has been reported worldwide wherever cereals are grown [3].

In addition to being pathogenic to plants, which may cause severe crop yield reduction, many *Fusarium* species are also capable of producing mycotoxins as secondary metabolites. Among these toxins are trichothecenes such as deoxynivalenol (DON), nivalenol (NIV), T–2 toxin, HT–2 toxin and diacetoxyscirpenol (DAS), zearalenone (ZEN), fumonisins and fusarins [4]. *Fusarium* toxins have been shown to cause a variety of toxic effects in both laboratory animals and livestock and on some occasions, they have been suspected to cause toxicity in humans [4]. Zearalenone is associated with hyperestrogenism in farm animals while trichothecenes have been linked to hemorrhagic syndrome and alimentary toxic aleukia in farm animals and humans, respectively [5]. *Fusarium* toxins are commonly encountered in cereals, maize, rice and beer [3]. Deoxynivalenol, ZEN and Fumonisins have been detected in Kenyan lager beers albeit at very low levels [6]. In Europe, Canada and USA , DON occurs widely in beer, while African native opaque beers derived from grains have been found to harbor Zearalenone, which is not common in clear beers [7]. Much of the documented work on occurrence of the *Fusarium* toxins in beer has been carried out in the temperate countries of Europe and North America and Asia. Presence of *Fusarium* toxins in clear beers could be as result of contaminated barley or other cereal adjuncts.

Barley is the main cereal subjected to malting as the principal raw material in brewing. The high moisture content at the germination stage provides a conducive environment for microbial proliferation [8]. Any attempt to chemically control fungal growth also interferes with the barley physiological and biochemical processes, hence reducing the malt quality. It also raises concern over chemical residues in the beer. Presence of *Fusarium* and other molds in barley has also been associated with ‘gushing’ (uncontrolled foaming) and off-flavor in beer [2, 8]. Therefore, barley destined for malting has to be of optimum microbial quality and free from mycotoxins.

Studies have shown that natural contamination of barley with toxin producing *Fusarium* strains can lead to *Fusarium* toxins in beer. The mycotoxin which best survives the brewing process is DON. One study using barley infected with *F. graminearum* found out that most of the DON (86%) in the naturally contaminated barley was rinsed off during steeping, but DON levels in the green malt after 5 days of germination increased by up to 114% of the levels in the original barley, kilning had little effect on DON levels, and 80–90% of DON was subsequently recovered in the finished beer [7]. Most of the ZEN in the malt has been found to end up in spent grain; however, ZEN, which finds its way into the wort is largely metabolized to β-zearalenol by brewing strains of *Saccharomyces cerevisiae* [7, 9].

The profile of secondary metabolites varies from one *Fusarium* species to another but variations within the species also exists. Secondary metabolites are an important tool when combined with morphological characteristics in *Fusarium* taxonomy [3].

Investigations in Kenya have revealed the presence of different species of *Fusarium* and fumonisin toxin in maize [10]. Deoxynivalenol producing *F. graminearum* strains have also been frequently isolated from wheat samples collected in the different wheat growing areas in Kenya [11,12]. Bearing in mind that barley growing also takes place in wheat production areas, the possibility of *Fusarium* infection in barley is high. This study investigated the occurrence of *Fusarium spp.* in Equatorial barley grain derived from two different regions in Kenya and stored grain that was ready for malting. The objective was to reveal the species of *Fusarium* that occur in barley, persistence of the inoculum till malting stage and their ability to produce mycotoxins. This was done with an overall goal of highlighting the potential threat posed by *Fusarium* to barley growing and brewing industry in Kenya.

MATERIALS AND METHODS

A total of twenty one samples consisting of newly delivered barley from two different regions (Olchoro–Mau escarpment region, and Timau–Mount Kenya region) and stored pre–steep grain ready for malting after the break of dormancy, were obtained from Kenya Maltings Limited, Nairobi. The freshly delivered samples were from pooled grain lots brought to the plant from each region. All the samples comprised visibly clean barley accepted for malting. Sample size was about 500g. After collection, the samples were stored under refrigeration at 5°C and plated for isolation of *Fusarium* within one week.

Isolation and Identification of *Fusarium*

Isolation was done by direct plating of barley seeds on homemade Potato Dextrose Agar (PDA). About 250 g of unpeeled baking grade, white skinned potatoes were autoclaved for 45 min in 500 ml of distilled water alongside 20 g of agar in 500 ml of water in a second flask. After straining through a cheese cloth, the potato broth was combined with the molten agar, topped to 1 liter, 20 g of glucose added and the mixture autoclaved for 15 min at 121°C [13]. Upon surface sterilization for 5 minutes with 10% commercial bleach (Jik®), active ingredient- sodium hypochlorite at 3.5%, in sterile distilled water, the barley kernels were rinsed twice with sterile distilled water, transferred to the solid media using sterile forceps and incubated at 25^{o}C for 7 days. Ten seeds were placed in each plate; all the experiments were carried out in duplicates. Daily observations were made to ensure that the colonies were isolated before spreading out. Level of contamination was expressed as percentage of samples found infected by *Fusarium* species after confirmation.

Identification was done by culturing the isolates on homemade PDA and Synthetic Nutrient agar (SNA) (KH_2PO_4 1.0g, KNO_3 1g, $MgSO_4.7H_2O$ 0.5g, KCL 0.5g, glucose 0.2g, saccharose 0.2g and agar 20.0g per liter of distilled water) for cultural and microscopic characteristic respectively [13, 14].

***In vitro* culturing of *Fusarium* isolates for toxin production**

Fusarium isolates were cultured on 50g of rice in 250ml conical flasks. Two moisture and temperature regimes were used: (a) According to Greenhalgh *et al*.[15], 20ml of distilled water were added to 50g of rice with a moisture content of 13.3% (determined using oven drying method [16]) to give an initial moisture content of 38.1%, the rice was capped with aluminum foil, allowed to set for 1 hour and then autoclaved for 30 min at 121°C. After cooling, the media was inoculated with a mycelium plug, 11 mm in diameter obtained with a sterile core borer from actively growing edge of a pure culture on PDA. The plug was placed at the center of the flask and pushed deep to ensure it contacted the moist rice. The cultures were recapped with sterile aluminum foil then incubated at 25°C in the dark for 35 days (b) 30ml of distilled water were added to 50g rice (same lot as used above) to give an initial moisture content of 45.8%; the same sterilization and inoculation procedure was followed as outlined above. The cultures were incubated at 25°C for 14 days and then transferred to 4°C for 21 days to induce toxin production as advised by Dr. John L. Richard, Romer Labs, Inc. USA [Personal Communication].

Fusarium toxins Analysis

Trichothecenes: Extraction of the cultures was done according to Association of Official Analytical Chemists (AOAC) method 986.18 for DON in wheat [17]. Column clean-up was performed using charcoal-alumina-celite columns. A mixture of both type A and type B trichothecenes standards (Sigma-Aldrich Chemicals Co., Germany) was also dissolved in Acetonitrile:Water (84:16) and treated same as the sample extract.

Thin Layer Chromatography (TLC)

A slight modification of AOAC method was done to accommodate concurrent analysis for Type A and B trichothecenes as described by the Dual Column Quantitative TLC Method for Type A and B trichothecenes -Method Code: tri–tl–01.00.2 (Romer® Labs, Inc. MO, USA) as outlined below.

(a) Type B trichothecenes (DON and NIV): The residue in the glass vial obtained from column clean-up and concentration was dissolved in 100 µl of acetone:methanol (2:1). Stoppered and vortexed for 30 seconds. 10 µl were spotted alongside DON and NIV standards on silica gel 60 precoated glass plates (0.25 mm layer thickness, E Merck, Darmstadt, Germany). The plates were developed in toluene: acetone (1:2) mobile phase. The plates were air dried and observed under long wave (366 nm) UV light for interfering compounds. They were then sprayed with 15% aluminum chloride in methanol, air-dried and heated at 150°C in a dry air oven until the standards were fully visible under UV-long wave 366 nm (Funa UV light, Model SL–800G, Japan). Finally, the plates were observed for presence of fluorescing spots with R_f values equivalent to the toxin standards.

(b) Type A trichothecenes (T–2 toxin, HT–2 toxin and DAS): The remaining solution from above was evaporated under a stream of nitrogen, dissolved in 90 µl of toluene:acetonitrile (97:3), stoppered and vortexed for 30 seconds. 10µl were spotted alongside T–2 toxin, HT–2 toxin and DAS standards on a reverse phase RP–18 precoated glass plates (0.25 mm layer thickness, E Merck, Darmstadt, Germany). The plates were developed in methanol:water:acetic acid (25:15:1) mobile phase, air-dried and observed for interfering compounds under long wave UV (366 nm). This was followed by spraying with 10% sulfuric acid in methanol, air-drying and heating at 150°C in a dry air oven until the standard spots were fully visible under long wave UV (366 nm). The TLC plates were observed for presence of fluorescing spots with R_f values equivalent to the toxin standards.

Deoxynivalenol confirmation and quantification: For the DON positive cultures, the acetonitrile:water extract was pre-treated with anhydrous ammonium sulphate to remove early eluting polar compounds [18]. The sample was then cleaned up in charcoal:alumina:celite column. Standards at five different concentrations were dissolved in acetonitrile:water (84:16) and treated the same as the sample extract to obtain a calibration curve. Analysis was done on High Pressure Liquid Chromatography (HPLC) under the following conditions: Column: RP–18 (0.5 µm) 4.6 mm x 250 mm column (E Merck, Darmstadt, Germany), Mobile Phase water:methanol:acetonitrile (90:5:5), Flow rate 0.9 ml min^{-1}: Shimadzu system controller SCL–6A, SPD–10A UV–Vis detector set at wavelength of 220 nm and range 0.0050 AUFS, LC- 6A pump and Column oven CTO–6A maintained at 40°C (Shimadzu Corporation, Japan).

Zearalenone: Screening of the cultures for zearalenone production was done by TLC according to AOAC official method 976.22 for zearalenone in corn [17]. Confirmation and quantification of zearalenone was done according to the HPLC method 985.18 of the AOAC for zearalenone in corn [17], under the following HPLC conditions; Column: RP–18 (0.5 µm) 4.6 mm x 250 mm column (E Merck, Darmstadt, Germany), Mobile phase: methanol:acetonitrile:water (1:1.6:2), flow rate 0.7ml min^{-1}: Shimadzu SCL–10A system controller, LC–10AS pump, Column oven CTO-10A maintained at 40°C, RF–1501 spectrofluorophotometer detector fitted with a flow cell, set at 236 nm (excitation) and 418 nm (emission) (Shimadzu Corporation, Japan).

RESULTS

Contamination of barley with *Fusarium*

Overall, seven out of the twenty one samples (33%) were contaminated with molds of *Fusarium* genus, 33.3% (n=9), 50% (n=4), 25% (n=8) of samples from Olchoro, Timau and stored pre-steep barley. *F.graminearum* and *F. poae* occurred concurrently on two samples originating from Olchoro in Mau Escarpment region. Only grain sourced from Olchoro was found to harbor *F. graminearum*. Two inconclusively

identified *Fusarium* isolates were obtained from barley grown in Timau. Stored grain was contaminated with *F. chlamydosporum* and *F. poae*.

Mycotoxins production by *Fusarium* isolates

The ability of the isolates to produce toxins on *in vitro* rice cultures and their mycotoxin profiles are illustrated on Table 1. Overall 5 out of 9 isolates were positive for the assayed mycotoxins. Deoxynivalenol was expressed by all isolates of *F. graminearum* and two strains of *Fusarium spp*. found in barley from Timau. ZEN was predominantly produced by *F. graminearum*. Confirmatory chromatographic profiles of the encountered mycotoxins are displayed on Figure 1 and 2.

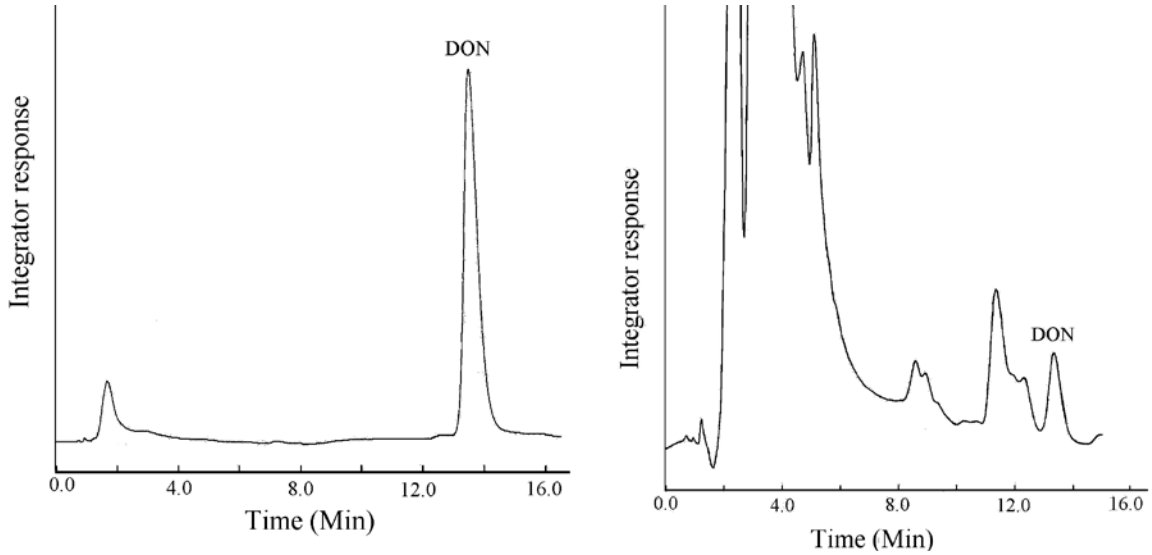

Figure 1: HPLC chromatogram profiles of Deoxynivalenol (DON) analysis
(a) Pure DON standard, retention time 13.5 min.
(b) Sample from *F. graminearum* culture extract

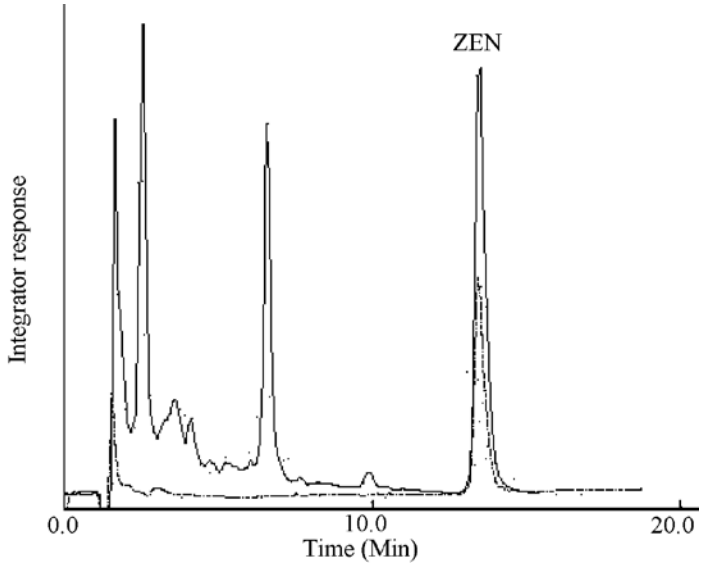

Figure 2: HPLC chromatogram profiles of Zearalenone (ZEN) Analysis Solid trace (*F. graminearum* culture extract), broken line (zearalenone standard). Zearalenone retention time (13.4 min), shown by the overlapping peaks in the figure.

Varying but appreciable amounts of DON and ZEN were produced by the toxigenic strains the rice cultures (Table 2). The culture with a lower moisture content of 38.1% accompanied by continuous incubation at 25^{o} C for 35days appeared to favor ZEN production by *F. graminearum* whereas a higher starting moisture content of 45.8% and subsequent transfer of the cultures to 4 ^{o}C after 14 days at 25^{o}C yielded comparatively higher levels of DON for the same isolates as demonstrated by mycotoxins concentration means. One strain of *F. graminearum* failed to produce detectable DON in rice cultures with lower moisture content and ZEN in the media with a higher moisture level. The HPLC method offered an extra confirmation step for the type of toxin in addition to quantification of the amount of toxins produced in the cultures.

DISCUSSION

The results confirm that *Fusarium* species does occur in barley growing locations where the pooled samples were sourced. Other investigators have encountered *Fusarium* in members of the *Gramineae* family grown around Kenya [10, 11, 12]. A study carried out in the wheat growing region of Nakuru District revealed that *F. poae, F. graminearum* and *F. chlamydosporum* were the most frequently isolated species in wheat and *F. verticilloides* in maize [11]. This exhibits the vulnerability of locally grown cereals including barley to *Fusarium*. *F. graminearum* and *F. poae* predominated in samples sourced from Olchoro which lies in the Mau Escarpment region. Olchoro has a warm climate and this probably favors *F. graminearum* compared to Timau region, which is cooler. *F. graminearum* is generally regarded as the most common species causing *Fusarium* head blight in warm regions of the world,

including parts of USA, Australia and Central Europe [1]. In Kenya, a variation of predominant *Fusarium* species in five wheat growing regions has been observed, *F. graminearum* dominated the warmer areas [12]. Changes in climatic conditions have also been shown to cause a shift in dominant *Fusarium* species found in wheat, barley, oats, rye and triticale during two consecutive cropping years in Netherlands [19]. Therefore, a more detailed field study in barley growing regions in Kenya, putting into consideration interacting factors such as location and climate would reveal the comprehensive prevalence and threat posed by *Fusarium*.

The isolation of *F. chlamydosporum* and *F. poae* from stored barley indicates that the inoculum carried over from the field can survive the storage period between harvesting to the break of dormancy when the grain is ready for malting. The high water activity created by malting conditions can then lead to a rapid proliferation of the molds and subsequently affect the quality of beer [8]. This necessitates the adoption of agronomical practices that minimize *Fusarium* infestation in the growing fields.

Screening and confirmatory tests demonstrated the presence of toxigenic species of *Fusarium* in barley. All the species encountered in this study are potential mycotoxin producers; however, some isolates did not produce detectable amounts of toxins in the rice cultures. *F. poae* has been shown to produce DAS, T–2 toxin, HT–2 toxin and NIV, whereas *F. chlamydosporum* produces moniliformin [3, 20]. The findings obtained in this study and those conducted by other investigators affirm that presence of toxin producing *Fusarium* in cereal crops could be widespread in Kenya. High levels of DON have been encountered in maize and wheat kernels in the wheat-growing region of Nakuru while Fumonisin B_1 was found to occur in maize from western Kenya [10, 11]. Wheat samples from several localities around Kenya have also been found to be tainted with T-2 Toxin [21].

CONCLUSION

This investigation forms the foundation of creating awareness on the possible threats posed by *Fusarium* to barley farmers, malting and the brewing industries in Kenya. Encountering of various species of the *Fusarium* from a limited sample of perceptibly acceptable grain confirms the ubiquitous nature of this mold in Equatorial barley. The occurrence of DON and ZEN producing *Fusarium* species warrants routine analysis of barley, which is the main raw material used in making of clear beers for molds and mycotoxins, DON in beer and ZEN in spent grain used as animal feed. More studies are required to assess the field prevalence within the various growing regions, extent of barley contamination with mycotoxins and post harvest survival rates of different *Fusarium* species encountered in barley kernels during storage to assess the risk posed by the mold at the malting stage. Such investigations would aid in formulating early warning mechanisms to prevent heavy losses if favorable conditions were to prevail. Additional investigations are planned as part of on-going work and other researchers are encouraged to join in.

Table 1: *In vitro* screening for mycotoxin production by *Fusarium* isolates in rice cultures

***Fusarium* code and strain**	**Source**	**DON**	**NIV**	**DAS**	**T–2 toxin**	**HT–2 toxin**	**ZEN**
1S (*F. graminearum*)	Olchoro	+	-	-	-	-	+
3S2 (*F. graminearum*)	Olchoro	+	-	-	-	-	+
5S1 (*F. graminearum*)	Olchoro	+	-	-	-	-	+
C (*F. clamydosporum*)	Storage	-	-	-	-	-	-
F (*F. poae*)	Storage	-	-	-	-	-	-
3S1 (*F. poae*)	Olchoro	-	-	-	-	-	-
5S2 (*F. poae*)	Olchoro	-	-	-	-	-	-
A (*Fusarium sp.*)	Timau	+	-	-	-	-	-
4F (*Fusarium sp.*)	Timau	+	-	-	-	-	-

(+) positive for the tested mycotoxin

(-) mycotoxin not detected

Isolates codes were derived from sample coding (labeled according to the holding Silos from which the kernels were sampled)

Table 2: Mean concentration (ng/g fresh wt. rice) of mycotoxins produced *in vitro* by *Fusarium* isolates in Rice Culture

Fusarium Strain	Deoxynivalenol ng/g		Zearalenone ng/g	
	A	**B**	**A**	**B**
F. graminearum strains				
1S	881[a]	900[a]	155(89)	177(52)
3S2	NA	506[a]	109[a]	ND
5S1	525	1032 (96)	1006(81)	213(77)
Fusarium spp.				
4F	884(20)	867(98)	ND	ND
A	951 (23)	659 (17)	NA	NA

ND- Not Detected, NA- Not analyzed, [a]- only one of the TLC positive cultures analyzed

Data in brackets indicate standard deviation of duplicate cultures analysis. Data in column A represents values from rice culture with initial moisture content of 38.1% incubated at 25°C for 35 days. Values in column B are from rice cultures with initial moisture content 45.8% incubated at 25°C for 14 days, then 4°C for 21 days. Detection limits: ZEN->12.5ng/g, DON->350ng/g

Isolates codes were derived from sample coding (labeled according to the holding Silos from which the kernels were sampled)

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