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**Research Article**

# Fumonisin $B_1$ ($FB_1$) and fumonisin $B_2$ ($FB_2$) levels in maize genotypes grown in Nakuru County-Kenya

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## Abstract

Maize (*Zea mays* L.) is a staple food crop for people in Kenya. It is usually contaminated by fungi especially Fusarium that produces mycotoxins, Fumonisins (FBs). This is a group of fungal toxins, occurring worldwide in maize infected by *Fusarium verticillioides.* Most common is Fumonisin $FB_1$ whose intake above 2 mg/Kg body weight/day plays a role in Neural tube defect and/or Oesophangeal cancer. There is no available data on distribution of different $FB_1$ production in various maize genotypes in Kenya. The objective of this study was to establish the levels of $FB_1$ and $FB_2$ in maize genotypes in Njoro and Molo Sub-Counties in Nakuru County, Kenya. Using purposive sampling, maize kernels showing no symptoms of Fusarium infection were collected from 277 farmers' stores in Molo and Njoro Sub-Counties. Fumonisin (FB) levels were determined using Liquid Chromatography Mass spectrometry (LC-MS). The levels of Fumonisin $B_1$ ($FB_1$) showed that H629 had 4437.53 µg/kg while H614 had the lowest 1315.7 µg/kg $FB_1$ levels. The levels of $FB_1$ in locations tested using t-test were significantly different ($P < 0.05$). Levels of Fumonisin $B_2$ ($FB_2$) was higher in maize genotype H629 which had 628.1 µg/kg. This finding reveals presence of Fumonisins (FBs) on symptomless maize kernels and map out the levels of FBs in maize genotypes. This is to enlighten the public and the agricultural officers on the maize genotypes and the levels of Fumonisins (FBs) in each genotype, hence the need to grow maize genotypes that will have the minimal levels of Fumonisins (FBs) for this area under the same environmental condition.

**Keywords:** *Fusarium verticillioides***,** Fumonisin $B_1$, Fumonisin $B_2$, Liquid Chromatography-Mass spectrometry (LC-MS)

## 1. Background

Maize (*Zea mays* L.*)* is one of the main staple food crops in Kenya with a production of 39.0 million (90 kg) bags in the year 2014 (Ministry of Agriculture and Livestock Economic Review 2015). In the same year, Nakuru County produced a total of 1,765,714 of 90kg bags from an area of 86,504 hectares of land. Kenya's maize production has been constrained by underlying factors such as soil acidification due to continuous use of Di-ammonium Phosphate (DAP) fertilizer, lack of access to improved and certified seeds, and due to the impact of maize diseases including Maize Lethal Necrosis Disease (MLND) and other diseases (Ministry of Agriculture and Livestock Economic Review 2015). The Government of Kenya (GoK) and the county

governments in the maize growing areas have initiated measures to increase yields including distribution of certified seeds and alternative fertilizers to farmers. The Kenya government is also implementing the second pilot phase of the Galana/Kulalu irrigation project in the North Coast region. In this second phase, production on a further five hundred thousand hectares was expected in the MY 2016/2017. In addition, GoK has also put up drying and storage facilities in the corn growing areas to reduce postharvest losses and limit aflatoxin contamination. In Nakuru County under Kenya Cereal Enhancement Programme (KCEP) program, farmers were supplied with maize seeds for planting and subsequently with gunny bags for storage.

Production of maize is highly constrained by ear rot fungi such as *Fusarium, Aspergillus, Penicillium* and *Sternocarpella* (Olanya *et al.*, 1997). Besides lowering the quality of maize, these fungal pathogens produce secondary metabolites (mycotoxins) that have detrimental effects on human and animal health (Van Egmond *et al.*, 2007). One of the secondary metabolites is *Fumonisin* $B_1$ ($FB_1$), a mycotoxin produced by *Fusarium verticillioides,* which causes equine leukoencephalomalacia, porcine pulmonary edema, human Oesophangeal cancer and liver cancer in animals (Thiel *et al.*, 1991). *Fusarium spp*. may also infect plants without showing ear rot symptoms (Munkvold and Desjardins, 1997). In addition, *Fusarium spp*., mainly *F. verticillioides* (Sacc.) Nurenberg and *F. proliferatum* (T. Matsushima) Nurenberg, are able to produce a wide array of structurally different mycotoxins, e.g Trichothecenes, fumonisins and Macrocyclic lactones (Rheeder *et al*., 2002; Munkvold 2003; Marin *et al*., 2013).

Fumonisins (FBs) are a group of mycotoxins produced by *F. verticilliodes* (Umma *et al*., 2006). In the natural environment, fumonisins are thought to be synthesized through the condensation of the amino acid alanine to an acetate-derived precursor. Branched-chain methyl groups are added at C-12 and C-16 by an *S*-adenosyl methionine transferase. The subsequent biosynthetic steps involving oxygenation and esterification of the acetate-derived backbone are yet unknown. It is not clear whether oxygenation and methylation occur before or after condensation with alanine. However, it appears likely that less oxygenated Trichothecenes such as $FB_2$, $FB_3$ and $FB_4$ are precursors of the more highly oxygenated $FB_1$. Fumonisins are similar in structure to the free sphingoid bases sphinganine and sphingosine that are found in sphingol-lipids. Structural similarity between free sphinganine and fumonisins led to the discovery that fumonisins are potent and specific inhibitors of the acyl CoA-dependent ceramide synthase (Wang *et al*., 1991).

Chemical structure of Fumonisin $FB_1$ ($FB_1$) is a 2S-amino-12S,16R-dimethyl-3S,5R,10R,14S, 15R-pentahydroxyeicosane with the C-14 and C-15 hydroxyl groups esterified by a terminal carboxyl group of propane-1,2,3-tricarboxylic acid while $FB_2$ is 10-deoxy $FB_1$ (Li *et al.*, 2012)
The biosynthesis of fumonisin and trichothecene, revealed that the gene cluster fumonisin biosynthetic gene (FUM in *Fusarium sp*. and *Aspergillus sp*.) is responsible for the production of fumonisins, (Alexander *et al*., 2009). Expression of these genes is co-regulated and is influenced by ecological conditions (Desjardins & Proctor, 2007). Production of fumonisin is dependent on FUM1 which expresses an enzyme complex known as polyketide synthase which catalyzes the initial step for fumonisin biosynthesis (Bojja *et al*., 2004). A positive correlation has been identified between the proportion of FUM1 transcripts estimated by real-time RT-PCR and proportion of fumonisins biosynthesized by *F. verticillioides* and *F. proliferatum* species (López-

Errasquín, *et al*., 2007). It was established that FUM19 lies at a distance of 35 kb downstream of the FUM1 gene that expresses an ATP-binding cassette responsible for exporting extracellular fumonisins (Proctor *et al*., 2003). The expression of an aminotransferase by FUM8 maintains biologically active and mature $FB_1$ molecule (Seo *et al*., 2001).

Conventional methods for detecting FBs have mainly involved chromatography and Enzyme linked immunoassay (ELISA). The most used chromatographic method is high performance liquid chromatography (HPLC) coupled with fluorescence detection, after pre-column derivatization with o-phthaldialdehyde (OPA) reagent (Bird *et al*., 2002). The detection of fumonisin using HPLC relied on derivatization of the analyte which has been simplified by establishing an automated pre-column derivatization method by using an autosampler (Kaltner *et al*., 2017).
To use ELISA technic, Since $FB_1$ is a small molecule with no immunogenicity, samples are conjugated to carrier proteins such as BSA (bovine serum albumin) or KLH (keyhole limpet hemocyanin) to generate immunogenicity (Ling *et al*., 2014). Studies by Burmistrova *et al*., (2014) used non-instrumental multi-detection of Fumonisin $B_1$ ($FB_1$), Ochratoxin A (OTA) and Zearalenone (ZEN) in cereal grains and silage. This test was based on a direct competitive enzyme immunoassay performed on a membrane. Ling *et al*., (2014) used conjugate $FB_1$-BSA to immunize Balb/c mice, and one hybrid cell line 4G5 excreting monoclonal antibody against $FB_1$. Fusing mouse Sp2/0 myeloma cells with spleen cells from the immunized mouse was injected with Hybridoma 4G5 into the abdomen of Balb/c mice, and harvested the anti-$FB_1$ mcAb. The results showed that anti-$FB_1$ mcAb was highly specific to Fumonisin $B_1$.

More chromatographic methods such as High-performance liquid chromatography tandem mass spectrometry, was used by Ren *et al*., (2011) who focused on clean up and optimization of extraction for determination of $FB_1$ and $FB_2$ residue in Maize. However, these techniques require the use of expensive apparatus, take a long time, and involve many skilled manipulations.

## 2. Materials and methods

### 2.1 Study site

This study was carried out in Njoro and Molo Sub-Counties of Nakuru County-Kenya in 2016.

### 2.2 Sample size determination

The study used a purposive sampling where maize samples from the enrolled maize farmers in Kenya Cereal Enhancement Programme (KCEP) supplied with different maize genotypes were sampled. Sample size determination was done according to Prasanna (2013) Sample size determination formula shown below;

$$SS = \frac{Z^2 * (p) * (1-p)}{C^2} = \frac{1.96^2 * (0.47) * (0.53)}{0.05^2} = 382.77$$

Where: Z = Z value (e.g. 1.96 for 95% confidence level), p = percentage incidence, expressed as decimal (.47 used for sample size needed), c = confidence interval, expressed as decimal, (e.g. 0 .04 = ± 4).

Since the population of the farmers was known (1000), the formula was subjected to a correction factor for a finite population; New SS = $\frac{SS}{1+\frac{SS-1}{pop}} = 277$ Where: pop = population
This resulted into: Sample size (SS) = 277 farmers. This sample of farmers was distributed amongst the two sub-counties (139 Molo and 138 Njoro farmers).

**2.3 Sampling procedure**

Sub-samples were taken from each of the farmers and a bulk sample based on the maize genotypes in each Sub-County (Molo and Njoro) was made. Fifteen grams of kernels (about 25 – 30 maize grains) which were 12 weeks Harvest Time Points After Physiological Maturity (HTPAPM), of each maize genotype showing no symptoms of fungal infection were sampled according to Alakonya *et al.*, (2004). Bulk samples were made in each Sub-County consisting of particular maize genotype. This gave five bulk samples from Njoro Sub-County and eight bulk samples depicting eight maize genotypes from Molo Sub-County.

**2.4 Maize Sample preparation for LC – MS**

Maize grain samples for LC-MS determination of levels of Fumonisins (FBs) were prepared using a method by Spanger *et al*., (2008). Dry samples 20g each of the 13 bulk samples were milled to fine powder using a laboratory mill and placed in a 100 ml conical flask. Samples in each conical flasks were then soaked with 100 ml acetonitrile/water (3:1 v/v), centrifuged for 4 hours at 155 rpm in a centrifuge (Micromeg MC-DS type). The samples were therefore first filtered through whatman No. 1 filter paper in two filtration cycles. The filtrate were further filtered through 0.45 µm Millipore filters followed by 0.22 µm Millipore filters. Using cuvettes, 20 µl of the filtrate were injected in LC-MS machine

**2.5 Water used in determination of levels of $FB_1$ and $FB_2$ in LC-MS**

Water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Methanol (Merck KGaA, Darmstadt, Germany) and acetonitrile (Labscan Limited, Dublin, Ireland) were HPLC-grade. Formic acid (Merck) was analytical reagent-grade. All chemicals were purchased from Sigma–Aldrich (Sigma-Aldrich Chemie B.V., Zwijndrecht, Netherlands). The standards used in the calibration were obtained from Cape Peninsula University of Technology, South Africa.

**2.6 LC–MS system setting used**

The LC–MS system consisted of a Waters Alliance 2695 separation module with a 100-ml Injection loop (Waters, direct - Q 3 UV Catridge, USA) coupled to a Quattro Ultima triple quadrupole mass spectrometer (Waters-Micromass, Manchester, UK) equipped with an electrospray interface. A 20-ml aliquot of the maize extracts were injected on an Agilent C18 (4.6 mm x 100 mm) column (Micromeg type MC-DS) at 30$^{o}$C column temperature. The gradient was composed of solvent A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.5ml $min^{-1}$. Spanjer *et al.*, (2008)

The mobile phase A was acetonitrile: methanol solution (50:50), B was aqueous formic acid (0.1%) and mobile phase B was, v/v). The flow rate used was 0.3 ml $min^{-1}$, the T column

temperature was 30ºC, and the injection volume was 20µL. Gradient elution program was according to Spanjer *et al.,* (2008) as shown in table 1.

**Table 1**: Gradient elution programme used in the instrument

| Step | Time (mins) | Solvent A (%) | Solvent B (%) | Flow (ml $min^{-1}$) |
|---|---|---|---|---|
| 1 | 0 | 90 | 10 | 0.3 |
| 2 | 12.0 | 30 | 70 | 0.3 |
| 3 | 16.0 | 30 | 70 | 0.3 |
| 4 | 17.5 | 10 | 90 | 0.3 |
| 5 | 20.0 | 90 | 10 | 0.3 |
| 6 | 21.0 | 90 | 10 | 0.3 |

Spanjer *et al.,* (2008).

The mass spectrometry detection was carried out in the electrospray ionization positive ion mode (ESI+) and multiple reaction monitoring (MRM) mode with a capillary voltage 3.0 kV, source block temperature at 120 ºC, a cone gas at 50 L $h^{-1}$, cone voltage of 45 V, a dessolvation temperature 350 ºC, gas 700 L $h^{-1}$ and 3.0 x $10^{-5}$MPa collision cell pressure. The determination of quantitative product ions (m/z) were at 352 and 336 and the collision energies (eV) are at 25 and 35 for $FB_1$ and $FB_2$ respectively. The qualitative product ions (m/z) were at 334 and 354, while the collision energies (eV) were at 35 and 30 for $FB_1$and $FB_2$ respectively.

### 2.7 Standard solutions of Fumonisin $B_1$ ($FB_1$) and Fumonisin $B_2$ ($FB_2$)

A stock used comprised of a mixture of 50.315 µg/g HPLC standard Fumonisin $B_1$ ($FB_1$) and 50.625 µg/g Fumonisin $B_2$ ($FB_2$) in vaporized form was obtained from Research Leader: Mycotoxicology Research Group Institute of Biomedical and Microbial Biotechnology, Cape Peninsula University of Technology, South Africa. The standards in a vial (contained a total Fumonisins of 100.940 µg). From this, a 100ml water: Acetonitrile (1:1) was added to make a standard solution of 1009.40 µg/L.

### 2.8 Data analysis

Total Fumonisins (FBs) levels in the maize genotypes were established by summation of levels of Fumonisin $B_1$ ($FB_1$) and Fumonisin $B_2$ ($FB_2$) in each of the maize genotypes. To establish the variation in the levels of fumonisins (FBs) in maize samples from the two Sub-Counties, two sample t-test was done at 95% confidence interval, using INSTAT computer program (University of Reading, UK).

## 3. Results

### 3.1 Total levels of Fumonisins (FBs) in the maize genotypes

The total levels of Fumonisins (FBs) in each of the maize genotype was recorded. Maize genotype that recorded the highest amount of Fumonisins (i.e. $FB_1$ + $FB_2$), was H629, which had a total FBs of 5065.63µg/kg. This was followed by maize genotype H626 which had total FBs of 3865.4 µg/kg, H624 (2850.73µg/kg), Pioneer (2767.78µg/kg), H6213 (2580.5µg/kg), H6210 (2505.7µg/kg) and H6218 (2316.98µg/kg). The lowest amount of FBs was recorded in H614 which had a total FBs of 1519.5µg/kg as shown in Figure 1.

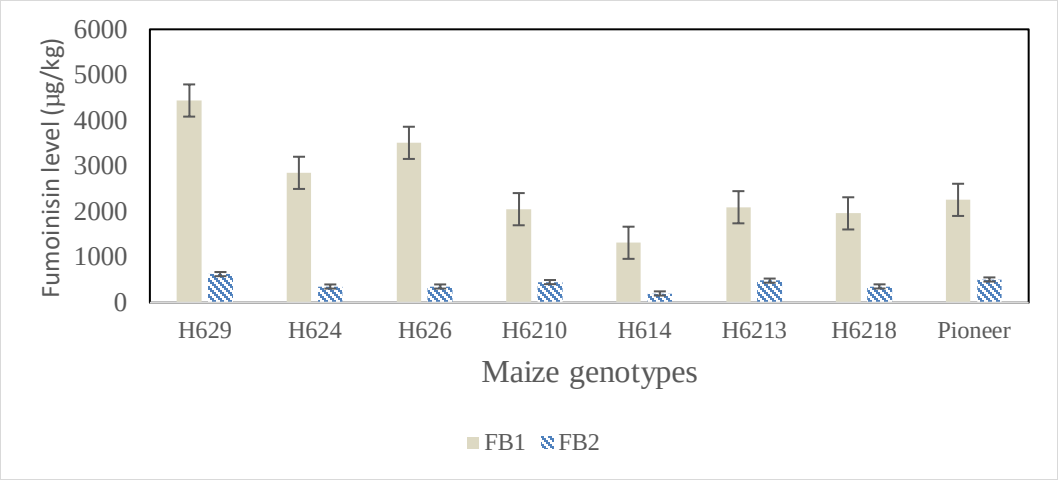


**Figure 1**: Fumonisins ($FB_1$ and $FB_2$) levels in maize genotypes

### 3.4 The variation between the levels of Fumonisin $B_1$ ($FB_1$) in maize varieties in Njoro and Molo Sub-Counties

Levels of $FB_1$ in Maize genotype H629 from Njoro (mean 6501 µg/kg) was significantly higher compared to the levels in H629 samples from Molo (mean 2374 µg/kg), t = 7.69, P = 0.005. Higher levels of $FB_1$ was recorded in Pioneer maize from Molo (mean 3469.1 µg/kg) compared to Pioneer from Njoro (mean 1046 µg/kg), t = 6.34, P = 0.024. It was however found that, $FB_1$ levels in H624 and H6218 samples from Njoro were significantly lower than in the samples of the same genotypes from Molo. Maize samples, H626, H6210 and H614 were only given out to farmers in Molo in this programme as shown in Table 2a. In table 2b, the maize genotypes where the levels of $FB_2$ were detected are shown. Fumonisin $B_2$ was not detected in some of the respective maize genotypes from the two Sub-Counties as shown in table 2b.

**Table 2a:** Levels of Fumonisin $B_1$ (µg/kg) in maize genotypes in (Molo and Njoro Sub-County)

| Maize variety | $FB_1$ levels Njoro | $FB_1$ levels Molo | T value | P - value |
|---|---|---|---|---|
| H629 | 6501b | 2374a | 7.69 | 0.005* |
| H624 | 1948a | 3754b | 7.91 | 0.004* |
| H626 | GNG | 3509 | - | - |
| H6210 | GNG | 2052.7 | - | - |
| H614 | GNG | 1315.7 | - | - |
| H6213 | 2062a | 2128.7a | 0.407 | 0.724 |
| H6218 | 1057.4a | 2865b | 20.17 | 0.002* |
| Pioneer | 1046b | 3469.10a | 6.34 | 0.024* |

GNG=Genotype Not Given out to farmers. Mean values in the same row denoted by similar letter are not significantly different at P≤0.05. Two sample t-test conducted at 95% CI.

**Table 2b:** Levels of Fumonisin $B_2$ (µg/kg) in maize genotypes in (Molo and Njoro Sub-County)

| Maize var. | $FB_2$ levels Njoro | $FB_2$ levels Molo |
|---|---|---|
| Pioneer | ND | 510.1 |
| H629 | ND | 628.1 |
| H 6213 | 485 | ND |
| H 6218 | 357.8 | ND |
| H 624 | 355.9 | ND |
| H 6210 | GNG | 453 |
| H 626 | GNG | 356.4 |
| H614 | GNG | 203.8 |

ND = Not detected, GNG = Genotype Not Given out to farmers.

## 4. Discussion

Liquid chromatography tandem mass spectrometry (LC–MS) method has successfully been used in the determination of levels of Fumonisins (FBs) in maize kernels after a single extraction, (Spanjer *et al.*, 2008). In this study, the levels of Fumonisin $FB_1$ ($FB_1$) detected in the different maize samples collected from the farmers in this study area were within the range previously reported by Kedera (1999) on poor quality maize from Western Kenya. The detected levels of $FB_1$ in this study is a cause for concern since the symptomless maize samples used were perceived to be fit for human consumption. The levels in each of the maize genotypes were higher than the levels set by WHO and FAO of 2 mg/kg body weight/day except for the levels in H614 (1519.5 µ/kg).

## 5. Conclusion

Symptomless maize kernels used for human consumption in Nakuru County, Kenya had high levels of Fumonisin $B_1$ ($FB_1$) compared to WHO/FAO maximum tolerable level of 2 mg/Kg body weight/day except for maize genotype H614 and H6218. However, the levels Fumonisin $B_2$ ($FB_2$) were below this set level.

Fumonisin $B_1$ ($FB_1$) was found in all the hybrid maize genotypes in Nakuru County, Kenya in 2015.

## Data availability

The Fumonisin levels data used to support the findings of this study are included in the article.

## Conflict of interest

The authors of this article declare that they have no conflict of interest


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