Nitrogen fertilization effect on chemical composition and contamination by fungal-fumonisin of maize kernels

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ABSTRACT

Due to rising domestic and global market demands, studies have been performed to obtain safe raw material with high nutritional quality. Therefore, the purpose of this study was to evaluate the effect of nitrogen (N) applied in topdressing (0, 80, 160 and 240 kg.ha^{-1}) in two key categories: 1) chemical composition (moisture, protein, lipid, ash, digestible starch, and total carbohydrates) and 2) fungi-fumonisin contamination, in maize kernels located in different areas of the cob (apex, middle and base). Field trials were conducted in the Southern Region of Brazil (Mauá da Serra, Paraná State), 2007-08 crop season, using randomized block design, with four replications. Analysis of variance (ANOVA) and an *a priori* significance level was set to determine significant differences using Tukey test's (p≤0.05). Incremental doses of nitrogen prompted significant changes in the chemical composition of the kernel, by increasing moisture, protein, lipid, and ash levels, while reducing digestible starch and total carbohydrate content (p<0.05). The deficiency of nitrogen (0 kg.ha^{-1}) led to higher infections of *Fusarium* sp., *Penicillium* sp., and yeasts, and contamination by fumonisins (FB$_1$ and FB$_2$) in the kernel (p<0.05). Kernels from the middle of cob presented better quality with lower infection by *Fusarium* sp. and yeast, as reduced contamination by fumonisins (FB$_1$ and FB$_2$; p<0.05).

Key words: bromatology, mycotoxin, sanity, Ureia, *Zea mays* L.

Efeito da fertilização nitrogenada na composição química e contaminação por fungo-fumonisina de grãos de milho

RESUMO

Frente as exigências de mercado e demanda mundial crescente, estudos são realizados objetivando obter matéria-prima segura e com alto valor nutricional. O trabalho objetivou avaliar efeito de nitrogênio (N) em cobertura (0, 80, 160 e 240 kg.ha^{-1}) em duas categorias chave: 1) composição química (umidade, proteína, lipídeo, cinzas, amido disponível e carboidratos totais) e 2) contaminação por fungo-fumonisina, em grãos de milho localizados em diferentes áreas da espiga (ápice, meio e base). O experimento a campo foi conduzido na Região Sul do Brasil (Mauá da Serra, Estado do Paraná), safra 2007/08, em delineamento de blocos casualizados com quatro repetições. Análise de variância (ANOVA) e Tukey test's (p≤0.05) foram utilizados para determinar diferenças significativas. Incremento nas doses de nitrogênio resultou em alterações na composição química do grão, aumentando o teor de umidade, proteína, lipídeo e cinza, e reduzindo o teor de amido disponível e carboidratos totais (p<0,05). A deficiência de nitrogênio (0 kg.ha^{-1}) propiciou maior infecção dos grãos por *Fusarium* spp., *Penicillium* spp. e leveduras, e contaminação por fumonisina (FB$_1$ e FB$_2$; p<0,05). Grãos do meio da espiga apresentaram melhor qualidade, com menor infecção por *Fusarium* spp. e leveduras, e contaminação por fumonisina (FB$_1$ e FB$_2$; p<0.05).

Palavras-chave: bromatologia, micotoxina, sanidade, ureia, *Zea mays* L.

Introduction

Brazil currently stands as the third largest producer of maize (*Zea mays* L.), with an estimated total production of 80 million tons for the 2016-17 crop season (USDA, 2016). Interestingly, unique climatic conditions allow Brazil to harvest maize in two different crop seasons every year. In the country, Paraná and Mato Grosso consistently produce impressive yields, which helps them remain the top two grain-producing states in the country. As projected for 2015-16 crop season, Paraná (21.2%-14.5 million tons) and Mato Grosso (23.3%-15.9 million tons) will be responsible for 44.5% of the national production (100%-68.5 million tons) (CONAB, 2016).

The nutritional composition of maize kernel, rich in carbohydrates (72% starch, dry basis), protein (9.5%, dry basis), fiber (9%, dry basis) and lipid (4.3%, dry basis), makes this cereal grain a staple food for both humans and animals in many countries (Paes, 2006).

Despite Brazilian maize's popularity, several viruses, bacteria, and fungi threaten its quality. Mycotoxins - particularly fumonisins produced by fungi of *Fusarium* genus - can reduce the nutritional quality of the crop (Blandino et al., 2008; Wordell-Filho & Spagnollo, 2013). Emphasis should be given to fumonisin B_1 (FB_1), the most abundant metabolites of this group of mycotoxins, representing approximately 70% of naturally contaminated foods and feeds (Shephard et al., 1996).

Oliveira et al., (2007), evaluating the effect of feeding quail (*Coturnix coturnix japonica*) with contaminated feed (FB_1-254.2 mg.kg^{-1}), observed a reduction in body weight (10.4%) and increase in hepatic weight (32.5%) of birds after 28 days of feed intake, compared to control group. In swine, the consumption of contaminated feed (FB_1-30 mg.kg^{-1}) resulted in pulmonary edema after 20 days, decreasing feed conversion and weight gain (Dilkin et al., 2004). Based on evidence, the International Agency for Research on Cancer (IARC) declared FB_1 toxins produced by *Fusarium* potential carcinogens (class 2B carcinogen) (IARC, 2002).

Soil fertility is one of the main factors responsible for the low productivity of areas destined for both grain yield and fodder. This fact is not only due to low levels of nutrients in soil, but also to the inappropriate use of fertilizers, particularly nitrogen and potassium, and high extraction capacity of maize crops (Coelho et al., 2002).

Essential for plants, nitrogen interferes in several features related to growth and development of the plant, which directly or indirectly affect the crop yield and quality. The influence of nitrogen fertilizer on maize grain quality has been reported by numerous authors, including its effects on: increase in protein, lipid, fiber and ash content (Shehzad et al., 2012); increase in protein and reduction in starch content (Holou & Kindomihou, 2011); reduction in contamination by FB_1 (Blandino et al., 2008); increase in infection by *Fusarium verticillioides* (Wordell-Filho & Spagnollo, 2013). However, the availability of studies assessing the influence of nitrogen in the nutritional quality and safety of maize kernels is still lacking. Furthermore, comparison between works may be biased since climatic conditions and cultivars used in experiments are different.

The purpose of this study was to evaluate the effect of nitrogen fertilization on chemical composition (moisture, lipid, protein, ash, digestible starch, and total carbohydrates) and contamination by fungal-fumonisin of kernels from different portions of maize cobs (apex, middle and base). Thus, we seek a better understanding of the effect that common cultural practices have on the quality of grain. Considering the demands of the market, segments of the supply chain should be directed to the production of food with high quality, which can be a factor determining the competitiveness in the international market.

Material and Methods

Experimental field trials were conducted during the 2007-08 crop season at a commercial farming operation in Mauá da Serra, located in Northern of Paraná State in the Southern Region of Brazil (23°58'S and 51°19'W), with an altitude average of 847 m.

The region has subtropical climate conditions, classified as Cfb according to Köppen, with average temperatures below 18 and 22 °C for the coldest and warmest month, respectively, and no defined dry season (Caviglione et al., 2000). During the experimental period, the average monthly precipitation levels were: October - 86 mm; November - 178.5 mm; December – 235 mm; January - 253.5 mm; February - 147.5 mm; March - 157 mm; Total - 1057.5 mm.

The soil for this location is classified as oxisol (Embrapa, 2006), left fallow for 30 years under a no-tillage system. The chemical characteristics of the soil to the depth of 0-20 cm are as follow: pH $CaCl_2$ of 4.63; 30.48 mg.dm^{-3} P (Mehlich^{-1}); 0.36 mg.dm^{-3} K$^+$; 3.26 cmol$_c$.dm^{-3} Ca^{+2}; 1.32 cmol$_c$.dm^{-3} Mg^{+2}; 0.46 cmol$_c$.dm^{-3} A^{+3}; and 40.71 g.kg^{-1} of soil organic matter.

A randomized block experimental design was employed in this study, consisting of four doses of nitrogen (0, 80, 160 and 240 kg.ha^{-1}) with four replications. The plots consisted of six rows, with 8.0 m of length and 0.7 m row spacing.

Seeding was performed using a mechanical planter adapted to a no-tillage system, in a density of five seeds per linear meter every 0.7 m. Based on the soil tests results, the fertilization at sowing was performed using a mixture containing 526 kg of super simple and 89 kg of urea per hectare.

All treatments, except the control, received 40 kg ha^{-1} of N at sowing. Seedling emergence occurred seven days after sowing. The application of the nitrogen-based topdressing was performed manually 25 days after emergence (DAE). Seeds of *Zea mays* L. (cv. Pioneer 30F53) were previously treated with insecticide (150 g.L^{-1} Imidacloprid and Thiodicarb 450 g.L^{-1}) at a dose of 1.5 liters per 100 kg of seed.

The middle four rows of each plot were harvested (160 DAE), ignoring 1.5 m at each end (border), and the cobs subjected to cleaning of husk with alcohol 70%, to minimize cross contamination of grains by pathogens from the surface. Then, the cob was divided into three equal parts (apex, middle and base), threshed by hand and dried at 40 °C for 24 hours (MOD 400, Nova Ética®, São Paulo, Brazil). After these procedures, the samples were grinded at 50 mesh and stored at -18 °C (MOD FE26, Electrolux®, Manaus, Brazil), until analysis.

The moisture content was determined by drying the sample for 12 hours/105 °C (MOD NV 1.5, Nevoni®, São Paulo, Brazil), it was then cooled in a desiccator and weighed (IAL, 2008). The protein content was determined by Kjeldahl, using a block digester (TE-MOD 40/25, Tecnal®, Piracicaba, Brazil) and a nitrogen distiller (MOD TE-036/1, Tecnal®, Piracicaba, Brazil). Thereafter, nitrogen was converted into protein - 6.25 factor (AOAC, 1995). Soxhlet extractors (TE-188 MOD, Tecnal®; MOD MA-487, Marconi®, Piracicaba, Brazil) were used to determine the lipid content by reflux with petroleum ether. After six hours of processing, the flask was removed from the extractor and dried at 105 °C/12 hours (1.5 MOD NV, Nevoni®, São Paulo, Brazil). Next, the flask was cooled at room temperature in a desiccator and weighed (AOAC, 1995). The ash content was determined by weighing the waste of plant after incineration (MOD 318D24, Quimis®, Diadema, Brazil) (IAL, 2008). Digestible starch content was determined by enzymatic hydrolysis and analysis in UV-visible measurements (MOD British S22, Biochrom®, Cambridge, United Kingdom) (Protocol PTF; Walter et al., 2005). The total carbohydrates were calculated by difference (% Total carbohydrates = 100% - (% Moisture + % Protein + % Lipid + % Ash). Analyses were done in duplicated or triplicated if necessary.

For mold and yeast count, 10 g of sample were homogenized into 90 mL of sterile peptone water 0.1% (v/v) and subjected to dilutions (10^{-1} to 10^{-6}), in tubes containing the same diluent. Each dilution (0.1 mL) was plated on potato dextrose agar (PDA), acidified to pH 4.0 with tartaric acid. After incubation at 25 °C/5 days (MOD TE-39, Tecnal®), colonies were counted and identified according to Samson et al. (1995).

Fumonisin determination was performed by High Performance Liquid Chromatography (HPLC), according to Ueno et al. (1993). The samples were analyzed in isocratic reverse phase HPLC (LC-10 AD pump and RF 535 fluorescence detector) using C18 Luna 5 µ column (4.6 x 250 mm), with wavelength of 335 nm and 450 nm for excitation and emission, respectively. The mobile phase consisted of methanol:sodium phosphate ($CH3OH:Na_2HPO_4$) 0.1M (80:20, v:v), adjusted with phosphoric acid to pH 3.3, flow 1 mL.min^{-1}. The detection limit of FB_1 and FB_2 were 27.5 and 35.3 ng.g^{-1}, respectively.

The data were subjected to analysis of variance (ANOVA), regression for nitrogen doses, and Tukey test's ($p \leq 0.05$), using STATISTICA version 6.0 (StatSoft Inc. ®, Tulsa, United States) and SISVAR version 4.0 (System for Analysis of Variance) (Ferreira, 2000). Molds and yeast counts were converted to logarithmic scale to reduce the range of the data values.

Results and Discussion

According to the data presented in Table 1 and 2, and depicted graphically in Figure 1, incremental applications of nitrogen in topdressing resulted in significant changes in chemical composition and fungal-fumonisin contamination of maize kernels. Moreover, differences were observed between kernels from different portions of the cob (apex, middle and base).

Nitrogen application in topdressing increased by 13.2% moisture content, with significant difference between higher doses of nitrogen (160 and 240 kg.ha^{-1}) and control (0 kg.ha^{-1}; $p<0.05$; Table 1), and positive linear correlations ($R^2=0.764$; $p<0.01$; Figure 1A). Since nitrogen availability extends the vegetative growth, due to delay in leaf senescence, immature grains with higher moisture content occur at harvest (Wingler et al., 2006).

Protein content in the kernel increased 34.5% when applying 240 kg.ha^{-1} of N, in comparison to the control (0 kg.ha^{-1}; $p<0.01$; Table 1). Moreover, protein content showed a positive linear correlation with nitrogen ($R^2=0.971$; $p<0.01$; Figure 1B).

Clearly, nitrogen is limiting for crop development and productivity, due to its importance in formation of amino acids, proteins, chlorophyll and essential enzymes, stimulating growth and development of shoot and root system (Holou & Kindomihou, 2011; Shehzad et al., 2012).

The findings from this study align with those of Farinelli & Lemos (2012). Evaluating the response of maize (cv. DKB 466) as a function of nitrogen application in topdressing (0-160 kg.ha^{-1}), in conventional and no-till systems, the authors observed a positive linear correlation between grain nitrogen content and incremental increases in fertilization (y=0.049x+14.10; $R^2=0.89$; $p<0.05$). The study observed an increase of 50.23% on the nitrogen content on the kernel, comparing control (0.kg ha^{-1}) to the highest nitrogen dose (160 kg.ha^{-1}).
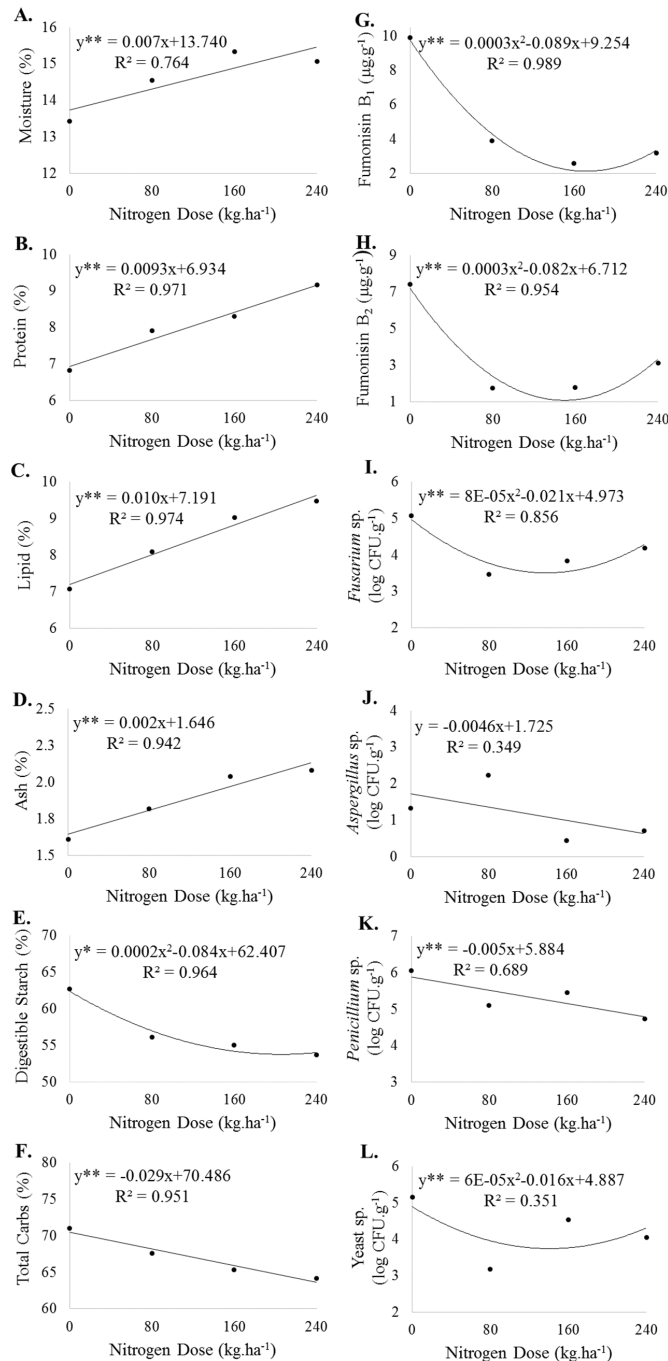
Table 1. Chemical composition and contamination by fungal-fumonisin of maize kernels (*Zea mays* L.; cv. Pioneer 30F53), in response to application of increasing dose of nitrogen (0-240 kg ha^{-1})

	Nitrogen dose (kg.ha^{-1})				*p*-value	CV%
	0	80	160	240		
Chemical Composition (%)						
Moisture	13.43 B	14.55 AB	15.33 A	15.07 A	0.005	7.33
Protein	6.82 C	7.91 B	8.30 B	9.17 A	0.000	6.86
Lipid	7.07 C	8.09 BC	9.03 AB	9.48 A	0.000	10.71
Ash	1.61 C	1.82 BC	2.04 AB	2.08 A	0.000	9.28
Digestible Starch	62.7 A	56.10 B	55.05 B	53.70 B	0.000	6.36
Total Carbs	71.06 A	67.63 B	65.30 C	64.19 C	0.000	2.60
Contamination (ug.g^{-1})						
Fumonisin B_1	9.39 A	3.39 B	2.07 B	2.70 B	0.000	47.54
Fumonisin B_2	6.93 A	1.23 B	1.27 B	2.61 B	0.000	48.86
Infection (log CFU.g^{-1})						
Fusarium sp.	5.07 A	3.47 B	3.84 AB	4.19 AB	0.010	22.78
Aspergillus sp.	1.33 A	2.23 A	0.44 A	0.71 A	0.320	182.03
Penicillium sp.	6.06 A	5.10 B	5.46 AB	4.73 B	0.005	13.47
Yeast	5.15 A	3.18 C	4.54 AB	4.05 BC	0.000	17.80

Carbs: Total carbohydrates; CV%: Coefficient of variation; Protein, lipid, ash, digestible starch and total carbohydrates results are expressed in dry-basis; Means followed by different letters indicate difference between lines (Nitrogen rates and parts of cob), by Tukey test's (p≤ 0.05).

Research has also been conducted on the connection of protein content and the vigor of seeds. For grasses, results showed that seed vigor is in fact associated with the protein content in the embryo, and may be enhanced by high concentrations of nitrogen in the soil (Carvalho & Nakagawa, 2000).

Application of 240 kg.ha^{-1} also resulted in 34.1 and 29.2% increases in lipid and ash content, compared to the control ($p<0.01$; Table 1). In addition, a positive linear correlation was observed between the components (lipid and ash) and the fertilizer (Lipid-$R^2=0.974$; Ash-$R^2=0.942$; $p<0.01$; Figure 1C and 1D, respectively).

Shehzad et al., (2012), evaluating the effect of nitrogen fertilizer (60-180 kg.ha^{-1}) on maize (cv. Safad), observed an increase in protein (+19.5%), lipid (+30.6%), and ash content (+22.3%) on the kernel through increasing the dose from 60 to 180 kg.ha^{-1} ($p<0.05$). According to the authors, lipid content, as well as minerals (ash), can be partly explained by the structural differences in the grain (i.e. endosperm and embryo weight) caused by nutrient availability.

A reduction in digestible starch (-14.3%) and total carbohydrate content (-9.7%) were observed by applying 240 kg ha^{-1} of N, compared to control ($p<0.01$; Table 1). Nitrogen doses presented a negative quadratic correlation with digestible starch ($R^2=0.964$; $p<0.05$; Figure 1E), and a negative linear correlation with total carbohydrates ($R^2=0.951$; $p<0.01$; Figure 1F).

Previous studies reported a negative relationship between protein and starch content, showing the results are not only linked to genetic factors, but also nitrogen availability. Nitrogen deficiency often result in the accumulation of non-structural carbohydrates (Kovacevic et al., 2012), leading to the accumulation of sugar by reducing the use of carbon skeletons for the synthesis of proteins (Wingler et al., 2006).

Almodares et al., (2009) obtained similar results evaluating the effect of crescent doses of nitrogen fertilizer on biomass (+16.76%), protein (+86.05%), carbohydrates (-28.09%) and soluble fiber content (-18.89%) of maize (cv. 704; 50-200 kg.ha^{-1} of urea). The results showed that the percentage of soluble carbohydrates decreased significantly from 17.8% using 50 kg.ha^{-1} to 12.8%, with 200 kg.ha^{-1}.

Although maize is a potential source of protein containing approximately 10% in dry matter, the grain is considered an energy food for humans and monogastric animals (Paes, 2006). Thus, the use of nitrogen fertilizer, in excess, would have a negative impact on the quality of maize, by reducing the primary energy reserves of grain, starch.

The results observed can also affect the physiological quality of seeds, since higher the energy stored greater will be the force provided to the plantlet during germination, affecting the initial growth of the plants and their ability to accumulate biomass (Lemoine et al., 2013).

Nitrogen also resulted in reduction of 71.2% and 62.3% grain contamination by FB$_1$ and FB$_2$, respectively, comparing the highest dose of nitrogen (240 kg.ha^{-1}) with control ($p<0.01$; Table 1). Additionally, contamination by fumonisin (FB$_1$ and FB$_2$) showed a quadratic correlation with nitrogen fertilization (FB$_1$-$R^2=0.989$; FB$_2$-$R^2=0.954$; $p<0.01$; Figure 1g and 1h, respectively). Using the quadratic equations, the maximum reduction of grain contamination by FB$_1$ (2.6 µg.g^{-1}) and FB$_2$ (1.1 µg.g^{-1}) would be obtained applying 148 and 137 kg.ha^{-1}, respectively.

Mycotoxin contamination (FB$_1$+FB$_2$=16.3 µg.g^{-1}) observed in the control (0 kg.ha^{-1}) were above the maximum tolerated limit for maize that will be submitted to further process (>5.0 µg.g^{-1}), as established by Resolution-RDC no. 7 of 18 February 2011 (Brazil, 2011). However, the condition in which the contamination was higher does not portray the reality, since nitrogen application in topdressing is a common practice aimed at increases in crop productivity.

* and ** significant at 5% and 1% by Student's t-test (linear and quadratic equation)

Figure 1. Chemical composition (a-f) and contamination (g-l) of maize kernels as response of nitrogen application in topdressing

The non-application of nitrogen (0 kg.ha^{-1}) also resulted in higher grain infections of *Fusarium* sp., *Penicillium* sp., and yeasts ($p<0.01$; Table 1). Nitrogen doses presented a quadratic effect with *Fusarium* sp. ($R^2=0.856$; Figure 1i) and yeast ($R^2=0.351$; Figure 1L), and a linear negative correlation with *Penicillium* sp. ($R^2=0.689$; Figure 1K) ($p<0.01$). Based on the quadratic equations, the maximum reduction of grain infection by *Fusarium* sp. (3.6 log CFU.g^{-1}) would be obtained by applying 132 kg.ha^{-1}.

Deficiency or excess of plant-available nitrogen can lead to stress, increasing their susceptibility to be attacked by pests and pathogens (Mcmahon, 2012). Blandino et al., (2008), observed a significant increase in the incidence ($p<0.05$) and severity ($p<0.01$) of diseases with non-applications of nitrogen fertilizer. In addition, the authors in this study observed that climatic conditions favored the emergence of diseases under excessive rainfall and mild temperatures during both flowering and ripening stages (Del Ponte et al., 2004).

As showed in Table 2, kernels from the base and middle of the cob presented higher moisture, protein, and ash content ($p<0.05$). Although, the differences observed are partly linked to grain development, since assimilates are not distributed evenly, being smaller grains formed at apex (Chen et al. 2013).

Fumonisin contamination (FB$_1$ and FB$_2$) was higher in kernels from base and apex of the cob ($p<0.01$), although contamination by FB$_2$ did not differ between the portions ($p>0.05$; Table 2). Moreover, contamination by FB$_1$ and FB$_2$ increased 857% and 686%, respectively, when compared contamination of kernels from middle and base of the cob. With significantly lower infection by *Fusarium* sp. and yeasts in the middle portion ($p<0.01$; Table 2).

Kikuti et al. (2003), also observed similar results, with greater fungal contamination in apex and base of maize cob. According to the authors, exposure of kernels from apex to temperature, pathogens and moisture, and accumulation of water in the base of cob, provides ideal conditions for fungal growth.

Table 2. Chemical composition and contamination by fungal-fumonisin of maize kernels (*Zea mays* L.; cv. Pioneer 30F53) located in different portions of cob (apex, middle and base)

	Area of Cob			*p*-value	CV%
	Apex	Middle	Base		
Chemical Composition (%)					
Moisture	13.96 B	15.27 A	14.56 AB	0.023	7.33
Protein	7.74 B	8.04 AB	8.38 A	0.032	6.86
Lipid	8.45 A	8.30 A	8.51 A	0.845	10.71
Ash	1.75 B	1.97 A	1.94 A	0.012	9.28
Digestible Starch	57.74 A	57.54 A	55.39 A	0.233	6.36
Total Carbs	68.10 A	66.43 A	66.61 A	0.055	2.60
Contamination (ug.g^{-1})					
Fumonisin B$_1$	4.82 B	0.79 C	7.56 A	0.000	47.54
Fumonisin B$_2$	3.79 A	0.59 B	4.64 A	0.000	48.86
Infection (log CFU.g^{-1})					
Fusarium sp.	5.10 A	2.99 B	4.34 A	0.000	22.78
Aspergillus sp.	0.33 A	1.35 A	1.86 A	0.231	182.03
Penicillium sp.	5.54 A	4.89 A	5.58 A	0.048	13.47
Yeast	4.90 A	2.98 B	4.80 A	0.000	17.80

CV%: Coefficient of variation; Means followed by different letters indicate difference between lines (Nitrogen rates and parts of cob), by Tukey test's (p≤ 0.05).

Conclusions

Incremental applications of nitrogen fertilizer in topdressing increased moisture (+12.2%), protein (+34.5%), lipid (+34.1%) and ash (+29.2%), and reduced digestible starch (-14.4%) and total carbohydrates content (-9.7%) in the kernels (cv. Pioneer 30F53), comparing the control (0 kg.ha^{-1}) with the highest dose of nitrogen (240 kg.ha^{-1}) (p<0.05).

Non-application of nitrogen (0 kg.ha^{-1}) resulted in higher contamination of kernels by fumonisin (FB$_1$ and FB$_2$), and infection by *Fusarium* sp., *Penicillium* sp. and yeast (p<0.05).

Kernels located in the middle of maize cob presented better quality, with lower contamination by fumonisin (FB$_1$ and FB$_2$), and infection by *Fusarium* sp. and yeast (p<0.05).

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