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Influence of macro- and micronutrient fertilization on fungal contamination and fumonisin production in corn grains

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Abstract

The aim of this study was to investigate the effects of nutrients (nitrogen, zinc and boron) on fungal growth and fumonisins production in corn samples obtained at the beginning of grain formation and at harvest. Three nitrogen doses were applied to the corn plants through soil in combination with three zinc doses and two boron doses during sowing. Mycological analysis of grains, using Dichloran Rose-Bengal Chloramphenicol Agar, collected at the beginning of formation demonstrated a fungal population predominantly of yeasts. Analysis of freshly harvested corn revealed a higher frequency of *Penicillium* spp. (72%) and *F. verticillioides* (27%). High Performance Liquid Chromatography analysis revealed that 100% of grains were contaminated with fumonisins B_1 at levels ranging from 0.3 to 24.3 mg/kg and 93% contaminated with fumonisin B_2 at levels ranging from 0.05 to 5.42 mg/kg. Nitrogen (50 kg/ha) in combination with boron (0.5 kg/ha) resulted in an increased fumonisin B_2 production.

Keywords: *Fusarium verticillioides*; Fumonisin; Corn; Micronutrients; Nitrogen

1. Introduction

Corn is one of the main Brazilian crops and is commonly employed in the human diet in its natural form as sweet corn or as a subproduct in bread, flour and dough, among others (Pinazza, 1993). In animal feeding, approximately 65% of corn grains are destined to the production of ration (National Trade of Animal Feed Industries, 2000).

Corn production and conservation methods able to counteract deteriorating fungi and mycotoxin producers are of great interest not only because of the intensive utilization of this crop but also because it is produced during a limited period of time and is supplied throughout the year. One of these methods is the adequate supply of minerals, both macro- and micronutrients, to the corn culture that protect against fungal attacks (Amézquita, Barrera, Arbeláez, Granada, & Ospira, 1993).

Nitrogen, the most important macronutrient, is a component of various protein molecules, enzymes, coenzymes, nucleic acids, and cytochromes (Büll, 1993). Excess nitrogen causes prolonged vegetative growth of the plant, rendering the leaves more exposed to pathogens, with the cell wall becoming thinner and vulnerable to fungal penetration (Reid, Zhu, & Ma, 2001).

Zinc is a micronutrient which exerts essential metabolic functions in the plant. It is a component of a large number of enzymes and is associated with the metabolism of carbohydrates, proteins and phosphate and with the formation of the structure of auxins, RNA and ribosomes (Borkert, 1989). Zinc deficiency renders the plants more susceptible to various diseases, many of them of fungal origin (Graham, 1983).

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Boron is another micronutrient that plays a role in the synthesis of the cell wall, nucleic acids (RNA and DNA) and phytohormones by the plants and also in the integrity of the plasma membrane (Yamada, 2000).

Fungal species responsible for the rotting of corn ears include *Fusarium verticillioides* (= *F. moniliforme*) and *F. subglutinans*, which cause loss and poor quality of grains, in addition to producing mycotoxins (Warfield & Gilchrist, 1999). The main mycotoxins produced by *F. verticillioides* are fumonisins (Scott, 1993), with fumonisin B_1 (FB_1), FB_2 and FB_3 being produced naturally by the fungus (Visconti & Doko, 1994). Among the fumonisins known, FB_1 is the most abundant and accounts for about 70% of the total concentration of fumonisin detected, followed by FB_2 and FB_3 (Norred, Plattner, & Chamberlain, 1993). Fumonisins cause leukoencephalomalacia in horses (Marasas et al., 1988), pulmonary edema and hydrothorax in swine (Harrison, Colvin, & Greene, 1990), and hepatotoxicity and hepatocarcinogenicity in rats (Gerderblom et al., 1988). Body weight and average daily weight gain have been shown to decrease in chicks in parallel with increasing dietary FB_1 (Ledoux, Brown, Weibking, & Rottingaus, 1992). In humans, FB_1 has been associated with esophageal cancer (Marasas, 1996; Rheeder et al., 1992; Sydenham et al., 1990). Based on toxicological evidence, the international agency for research on cancer (IARC) has established that FB_1 is potentially carcinogenic (class 2B) to humans (IARC, 1993).

The supply of adequate amounts of nutrients to the corn culture is fundamental for the adequate development of the plant; however, deficiency or excess of essential nutrients may influence the incidence of diseases caused by fungi as well as the production of mycotoxins in grains. Thus, the aim of this study was to investigate the effects of nutrients (nitrogen, zinc and boron) on fungal growth and fumonisins production in corn samples obtained at the beginning of grain formation and at harvest.

2. Material and methods

2.1. Study place

The study was conducted at the experimental station of Escola Superior de Agricultura Luiz de Queiroz – ESALQ-USP, municipality of Piracicaba, SP, Brazil.

2.2. Corn hybrid

The Cargill hybrid 909 was used for planting. Seeds were sowed on December 9, 1999 using a direct planting system.

2.3. Experimental design

The experimental design was a random block consisting of three replicates and treatments in a $[(3 \times 3) + 3]$ factorial scheme, corresponding to 3 nitrogen (N) and 3 zinc (Zn) doses with 3 additional treatments corresponding to nitrogen (N) and boron (B) (treatments 4, 8 and 12), for a total of 12 treatments. The 12 treatments are indicated in Table 1.

Table 1
Description of the different treatments

Treatment	Nitrogen	Discrimination
1	(0 kg/ha)	Zinc (0 kg/ha) and Boron (0 kg/ha)
2		Zinc (0.5 kg/ha)
3		Zinc (1.0 kg/ha)
4		Boron (0.5 kg/ha)
5	(50 kg/ha)	Zinc (0 kg/ha) and Boron (0 kg/ha)
6		Zinc (0.5 kg/ha)
7		Zinc (1.0 kg/ha)
8		Boron (0.5 kg/ha)
9	(100 kg/ha)	Zinc (0 kg/ha) and Boron (0 kg/ha)
10		Zinc (0.5 kg/ha)
11		Zinc (1.0 kg/ha)
12		Boron (0.5 kg/ha)

The macronutrient (nitrogen) and micronutrients (zinc and boron) were applied to the soil during sowing. The sources used were urea, zinc sulfate and boric acid. Three doses of nitrogen (0, 50 and 100 kg/ha) were applied in combination with zinc (0, 0.5 and 1.0 kg/ha) and boron (0 and 0.5 kg/ha) as illustrated in Table 1.

Each plot was 5.0 m long and 3.2 m wide, for a total area of 16 m^2. Sowing was performed manually, with nine seeds being distributed per linear row meter, with the experimental plot consisting of four 5-m rows spaced 0.8 m apart. On the occasion of sowing, 100 kg/ha phosphorus, 40 kg/ha potassium and 30 kg/ha nitrogen (except for the treatment in which no nitrogen was added) were applied. The sources used were simple superphosphate containing 18% P_2O_5, potassium chloride containing 60% K_2O, and urea containing 45% nitrogen.

2.4. Corn sampling

Corn ears were collected on February 24, 2000, at the beginning of the milky grain stage (75 days after sowing), and on April 24, 2000 during harvest (135 days after sowing). Four silks were collected for each treatment and subdivided into two portions, for a total of 144 corn samples, with 72 samples being collected at the beginning of the milky grain stage (75 days after sowing) and 72 at the time of corn harvest (135 days after sowing).

2.5. Count, isolation and identification of the mycoflora in corn grains

One subsample of about 30 g was taken from each corn sample and disinfected by immersion in 2% sodium hypochlorite solution for 3 min, followed by three rinses with sterile distilled water. From this subsample, 33 samples were randomly taken and sown on Petri dishes containing Dichloran Rose-Bengal Chloramphenicol Agar (11 kernels per dish) (Pitt, King, & Hocking, 1979). The plates were

incubated at 25 °C for 5 days and the results are reported as the percentage of grains infected with fungi. The fungal colonies were counted and identified using the morphological criteria of Nelson et al. (Nelson, Touson, & Marasas, 1983), Pitt and Hocking (1997) and Arx (1974).

2.6. Determination of fungi in atmospheric air (Gambale, Purchio, & Paula, 1983)

The presence of fungi in air was determined at the beginning of the milky grain stage (1st collection) and corn harvest (2nd collection) by exposing 10 Petri dishes containing Sabouraud dextrose agar (Oxoid) supplemented with 100 μg/ml chloramphenicol for 15 min. After exposure, the plates were incubated in an oven at 25 °C for a maximum of 7 days. The fungal colonies were counted and identified using the morphological criteria of Nelson et al. (1983), Pitt and Hocking (1997) and Arx (1974).

2.7. Determination of fumonisins in corn grain samples (Sydenham, Shephard, Thiel, Snijman, & Stockenstrom, 1996)

Fifty grams of triturated corn was transferred to a glass flask, 100 ml of a methanol (Synth) and water solution (3:1, v/v) was added, and the mixture was homogenized in a shaker for 30 min. The extract was filtered through Whatman No. 4 filter paper and the pH was adjusted to 5.8–6.5 with 1 N NaOH using a pHmeter (Hanna-HI-8519). A 10-ml aliquot of the filtrate was purified on a minicolumn containing 500 mg ion-exchange silica (500 mg/10 ml, BondElut SAX, Varian), preconditioned with 5 ml methanol and 5 ml methanol:water (3:1, v/v). The cartridge was washed with 5 ml methanol:water (3:1, v/v) and 3 ml methanol. Fumonisins were eluted with 10 ml methanol:acetic acid (99:1, v/v) (Synth). The columns were conditioned and washed at a flow rate of less than 2 ml/min and sample application and toxin elution were performed at a flow rate of less than 1 ml/min controlled with a VAC-ELUT apparatus (Varian) coupled to a vacuum pump (Olidef-CZ). The extracts were filtered through a GVWP-01300 membrane (Millipore) and concentrated to dryness at 60 °C. The toxins were resuspended in 1 ml acetonitrile:water (70:30, v/v) (Synth) and stored in amber flasks.

Fumonisins were quantified in 100 μl of the sample by derivatization with 200 μl OPA (40 mg ortho-phthaldialdehyde dissolved in 1 ml methanol, 5 ml 0.1 M sodium tetraborate and 50 μl 2-mercaptoethanol). After 2 min, 20 μl of the reaction product was injected into a Shimadzu LC-10AD high-performance liquid chromatograph (HPLC) equipped with an RF-10 AXL fluorescence detector, using a 5 ODS-20 C_{18} column (150 × 4.6 mm; Phenomenex, Ultracarb) maintained at 30 °C. The excitation and emission wavelengths were 335 and 440 nm, respectively. Acetonitrile:water (1:1) with 0.5% acetic acid was used as the mobile phase and fumonisins were eluted at a flow rate of 1 ml/min. The detection limit of the method was 50 μg/kg for FB_1 and FB_2. Recovery data were obtained in triplicate by fortifying the corn sample with 0.5 mg/kg FB_1 and FB_2. The average recovery was 108% for FB_1 and 87.4% for FB_2.

2.8. Determination of water activity

Water activity of the corn grain was determined by automatic analysis using the Aqualab CX-2 apparatus (Decagon Devices Inc., Pullman, Washington).

2.9. Climatological data

Climatic data (temperature, relative air humidity and rainfall) comprising the period from December 1999 to April 2000 were obtained from the climatology sector of the Department of Exact Sciences, Esalq, Piracicaba, and are available at ce.esalq.usp.br.

2.10. Statistical analysis

The data obtained for each parameter evaluated were submitted to analysis of variance by the F test. To fulfill the assumptions of the mathematical model, the relative frequencies of contamination with *F. verticillioides* were transformed to $\ln(x+1)$ and the FB_1 and FB_2 concentrations present in corn grains were transformed to $1/x$ and $\ln(x+1)$. For quantitative factors, polynomial regression analysis at the 5% level was performed to choose the model that would best fit the results. Correlations were established between the variables water activity, relative frequency of the fungi (*F. verticillioides* and *Penicillium* spp.) and FB_1 and FB_2. The statistical analyses were performed with the Statistical Analysis System program (SAS, 1996).

3. Results and discussion

The corn samples collected at the beginning of the milky grain stage (75 days after sowing) exhibited the following mycoflora: yeasts (29%), *Cephalosporium* spp. (6%), *Aspergillus niger* (4%), *F. Verticillioides* (4%), *Cladosporium* spp. (3%), nonsporulated fungi (3%), *Penicillium* spp. (3%), *Fusarium equiseti* (3%), *A. flavus* (3%), *Mucor* spp. (3%), *Rhizopus* spp. (3%), *Alternaria* spp. (3%), *Scopulariopsis* spp. (3%), and *Drechslera* spp. (3%) (Table 2). Analysis of freshly harvested corn samples (135 days after sowing) revealed the presence of the following mycoflora: *Penicillium* spp. (72%), *F. verticillioides* (27%), *Cephalosporium* spp. (24%), nonsporulated fungi (14%), yeasts (12%), *Basipetospora* spp. (6%), *A. flavus* (5%), *A. niger* (3%), *Cladosporium* spp. (3%), *Alternaria* spp. (3%), *Scopulariopsis* spp. (3%), and *Nigrospora* spp. (3%) (Table 3).

The high frequency of yeasts and low frequency of *F. verticillioides* at the beginning of the milky grain stage has also been demonstrated in Brazil by Almeida et al. (Almeida

Table 2
Relative frequency (%) of contaminated grains collected at the beginning of the milky grain stage (75 days after sowing)

Treatment 1 N(0) + Zn(0)+B(0)	Treatment 2 N(0) + Zn(0.5)	Treatment 3 N(0) + Zn(1.0)	Treatment 4 N(0) + B(0.5)
Yeast (26%)	Yeast (30%)	Yeast (38%)	Yeast (34%)
Cephalosporium spp. (9%)	*F. verticillioides* (3%)	*Cladosporium* spp. (4%)	*Cephalosporium* spp. (9%)
Aspergillus niger (3%)	*Aspergillus niger* (3%)	*F. verticillioides* (3%)	*Aspergillus niger* (4%)
Cladosporium spp. (3%)	*Cladosporium* spp. (3%)	*Aspergillus niger* (3 %)	*Aspergillus flavus* (3%)
NSF (3%)	*Cephalosporium* spp. (3%)	*Cephalosporium* spp. (3%)	*Cladosporium* spp. (3%)
	Mucor spp. (3%)	*Mucor* spp. (3%)	*Penicillium* spp. (3%)
		Scopulariopsis spp. (3%)	*Scopulariopsis* spp. (3%)
		Alternaria spp. (3%)	*Rhizopus* spp. (3%)
Treatment 5 N(50) + Zn(0) + B(0)	Treatment 6 N(50) + Zn(0.5)	Treatment 7 N(50) + Zn(1.0)	Treatment 8 N(50) + B(0.5)
Yeast (17%)	Yeast (14%)	Yeast (31%)	Yeast (43%)
F. verticillioides (3%)	*Aspergillus niger* (9%)	*Cladosporium* spp. (3%)	*Cladosporium* spp. (3%)
Aspergillus niger (3%)	*Cladosporium* spp. (3%)	*Cephalosporium* spp. (3%)	*Rhizopus* spp. (3%)
Cladosporium spp. (3%)	*Penicillium* spp. (3%)	*Penicillium* spp. (3%)	*Penicillium* spp. (3%)
Penicillium spp. (3%)	*Mucor* spp. (3%)	NSF (3%)	NSF (3%)
	Drechslera spp. (3%)		
Treatment 9 N(100) + Zn(0) + B(0)	Treatment 10 N(100) + Zn(0.5)	Treatment 11 N(100) + Zn(1.0)	Treatment 12 N(100) + B(0.5)
Yeast (36%)	Yeast (41%)	Yeast (31%)	Yeast (11%)
Cladosporium spp. (4%)	*F. verticillioides* (9%)	*Cephalosporium* spp. (9%)	NSF (6%)
F. verticillioides (3%)	*Cladosporium* spp. (5%)	*Cladosporium* spp. (4%)	*Cladosporium* spp. (4%)
Aspergillus niger (3%)	*Penicillium* spp. (3%)	*F. equiseti* (3%)	*F. verticillioides* (3%)
Rhizopus spp. (3%)		*Penicillium* spp. (3%)	*Scopulariopsis* spp. (3%)
Mucor spp. (3%)		*Mucor* spp. (3%)	
Scopulariopsis spp. (3%)		*Scopulariopsis* spp. (3%)	
NSF (3%)		NSF (3%)	
Drechslera spp. (3%)			

NSF = nonsporulated fungi.

Table 3
Relative frequency (%) of contaminated grains obtained from freshly harvested corn (135 days after sowing)

Treatment 1 N(0) + Zn(0) + B(0)	Treatment 2 N(0) + Zn(0.5)	Treatment 3 N(0) + Zn(1.0)	Treatment 4 N(0) + B(0.5)
Penicillium spp. (70%)	*Penicillium* spp. (72%)	*Penicillium* spp. (57%)	*Penicillium* spp. (76%)
Cephalosporium spp. (28%)	*Cephalosporium* spp. (40%)	*F. verticillioides* (45%)	NSF (48 %)
Yeast (26%)	*F. verticillioides* (31%)	*Cephalosporium* spp. (30%)	*F. verticillioides* (21%)
NSF (22%)	NSF (7%)	*Aspergillus flavus* (10%)	*Cephalosporium* spp. (17%)
F. verticillioides (10%)	*Scopulariopsis* spp. (3%)	Yeast (9%)	Yeast (12%)
Cladosporium spp. (3%)		NSF (4%)	
Alternaria spp. (3%)		*Nigrospora* spp. (3%)	
Treatment 5 N(50) + Zn(0) + B(0)	Treatment 6 N(50) + Zn(0.5)	Treatment 7 N(50) + Zn(1.0)	Treatment 8 N(50)+B(0.5)
Penicillium spp. (67%)	*Penicillium* spp. (73%)	*Penicillium* spp. (81%)	*Penicillium* spp. (77%)
F. verticillioides (23%)	*Cephalosporium* spp. (31%)	*Cephalosporium* spp. (32%)	Yeast (27%)
Cephalosporium spp. (16%)	NSF (25%)	*F. verticillioides* (31%)	*F. verticillioides* (26%)
NSF (13%)	*F. verticillioides* (25%)	NSF (7%)	*Cephalosporium* spp. (8%)
Yeast (10%)	*Aspergillus flavus* (7%)	*Cladosporium* spp. (3%)	NSF (4%)
Basipetospora spp. (6%)	Yeast (6%)		*Alternaria* spp. (3%)
Nigrospora spp. (3%)			*Aspergillus niger* (3%)
Treatment 9 N(100) + Zn(0) + B(0)	Treatment 10 N(100) + Zn(0.5)	Treatment 11 N(100) + Zn(1.0)	Treatment 12 N(100) + B(0.5)
Penicillium spp. (87%)	*Penicillium* spp. (67%)	*Penicillium* spp. (76%)	*Penicillium* spp. (65%)
F. verticillioides (23%)	*F. verticillioides* (36%)	*F. verticillioides* (31%)	*Cephalosporium* spp. (37%)
Cephalosporium spp. (23%)	*Cephalosporium* spp. (27%)	NSF (22%)	*F. verticillioides* (24%)
NSF (3%)	Yeast (17%)	*Cephalosporium* spp. (10%)	*Aspergillus flavus* (3%)
Yeast (3%)	NSF (6%)	Yeast (3%)	
Alternaria spp. (3%)	*Aspergillus flavus* (3%)	*Aspergillus flavus* (3%)	
Cladosporium spp. (3%)	*Aspergillus niger* (3%)	*Aspergillus niger* (3%)	
		Cladosporium spp. (3%)	

NSF = nonsporulated fungi.

et al., 2002) who analyzed samples from the Capão Bonito region. In Argentina, Chulze, Ramirez, and Farnochi (1996) also reported a low frequency of the fungus 45 days after flowering. The low relative frequency of these fungi

during this phase might be due to the lack of substrate necessary for the growth of these toxigenic fungi. This phase is known to be the initial stage of accumulation of starch in the endosperm of grains (Fancelli & Dourado-Neto, 1999). The low frequency of *F. verticillioides* was responsible for the lack of detectable levels of fumonisins. This agrees with results reported by Scussel and Rodrigues-Amaya (1986) and Almeida et al. (2002) who analyzed grain samples obtained from freshly harvested corn. Chulze et al. (1996), 45 days after flowering, observed low levels of fumonisins due to the low frequency of *F. verticillioides*. These results demonstrate that during the first collection the application of nitrogen, zinc or boron to corn did not significantly influence the growth of *F. verticillioides*, *A. flavus* or *Penicillium* spp., the main mycotoxin-producing species. The water activity of grains collected at the beginning of the milky grain stage (75 days after sowing) ranged from 0.94 to 0.98. In February, the time of the first collection, the mean maximum and minimum temperatures were 29.9 and 19.2 °C, respectively, whereas mean relative humidity was 87% and mean rainfall was 4.3 mm.

The high frequency of *Penicillium* spp. observed in the second sampling (freshly harvested corn) may be due to the type of corn hybrid, the study region, environmental conditions, and interactions between fungal species, as well as to the water activity levels of the corn grains. In Brazil, other investigators also reported a higher frequency of *Penicillium* spp. compared to *F. verticillioides* in freshly harvested corn (Almeida et al., 2002; Castro, Soares, & Furlani, 1995). The reduced water activity levels in the grains (0.77–0.91), the mean temperature of 22.0 °C, the mean relative humidity of 72% and mean rainfall of zero in April, the period of the second sampling, are factors that may also explain the higher frequency of the genera *Penicillium* and *Fusarium*. According to Lacey et al. (Lacey, Ramakrishna, Hamer, Magan, & Marfleet, 1991), some species regarded as storage fungi are characterized by pre-harvest colonization. The minimum water activity necessary for the growth of some *Penicillium* species ranges from 0.78 to 0.83 and the temperature optimum ranges from 20 °C and 26 °C. On the other hand, *F. verticillioides*, a field fungus, grows on substrate with a minimum water activity of 0.87 and at an optimum temperature range of 22–28 °C (Lacey et al., 1991). It should be emphasized that in Brazil several authors cite *Penicillium* spp. as the most frequent fungus in soil samples collected in corn plantation regions (Almeida et al., 2002; Zorzete, 2004).

Regression analysis showed a positive linear effect of zinc concentration on the relative frequency of *F. verticillioides* ($p < 0.05$, $r^2 = 0.94$), i.e. the higher the zinc concentration (0, 0.5 and 1.0 kg/ha), the higher the relative frequency of *F. verticillioides* (18.6, 30.9 and 36.1%, respectivamente). In addition, Pearson's correlation coefficient showed a positive correlation ($p < 0.05$) between FB_2 production and the relative frequency of *F. verticillioides*, i.e. the higher the frequency of *F. verticillioides*, the greater the production of FB_2. Ono et al. (2002) also demonstrated that an increase in the frequency of *F. verticillioides* (10^6 CFU/g) led to an increase in fumonisin production. Other investigators also observed an increase in the frequency of *Fusarium* spp. in zinc-supplemented culture (Amézquita et al., 1993; Cuero, 2000). A plant supplemented with increasing zinc concentrations may provide this micronutrient for the development of *F. verticillioides* or may increase the metabolism of carbohydrates and proteins in grains, which in turn serve as a substrate and growth factor for fungi (Borkert, 1989).

F. Verticillioides showed a negative correlation with *Penicillium* spp. ($p < 0.05$) in practically all treatments. This correlation was more evident in the case of the N(0)Zn(1.0) treatment, in which the relative frequency of *F. verticillioides* was increased, whereas the frequency of *Penicillium* spp. was reduced. This negative correlation between *F. verticillioides* and *Penicillium* spp. probably indicates a competitive interaction, and has also been reported by Marin, Sanchis, Arnau, Ramos, and Magan (1998) who analyzed irradiated corn grains inoculated with *Penicillium* species and *F. verticillioides*. These authors observed that *P. implicatum* grew faster than *F. verticillioides* at a water activity of 0.95–0.98. In addition, the growth of *Fusarium* spp. was inhibited by *Penicillium* species in corn grains maintained at 25 °C. In natural situations, more vigorous species have been reported to inhibit the growth of other species that are present in the same niche (Horn & Wiclow, 1983). Environmental stress is also an important factor in the competition between microorganisms.

The nonsignificant effects of nitrogen on the frequency of *F. verticillioides* agree with the results reported by Ramirez et. al (Ramirez, Torres, Rodriguez, Castillo, & Chulze, 1997).

In the first sampling, at the beginning of the milky grain stage, the following wind-dispersed fungi were found: *Fusarium* spp. (100%), *Cladosporium* spp. (80%), *Trichoderma* spp. (50%), *Aspergillus* spp. (40%), and *Penicillium* spp. (40%). In the second sampling, the airborne contaminants detected were: *Cladosporium* spp. (100%), *Drechslera* spp. (100%), *Aspergillus* spp. (70%), *Epicoccum* spp. (70%), *Penicillium* spp. (60%), *Mucor* spp. (60%), *Alternaria* spp. (40%), *Fusarium* spp. (30%), *Nigrospora* spp. (30%), and nonsporulated fungi (10%).

Analysis of the trend of corn contamination with wind-dispersed fungi during the different stages of plant maturation showed that *Fusarium* spp. was the prevalent genus in the first stage, i.e. 75 days after sowing. This frequency differed from that found by Almeida et al. (2002) who analyzed the frequency of wind-dispersed fungi at different maturity stages in Ribeirão Preto and Capão Bonito, Brazil. The authors observed a predominance of *Fusarium* spp. in the middle and late stages (105–135 days after flowering). It should be noted that the wind-dispersed fungi isolated in our experiment are considered to be universal dominants. In Brazil, a high frequency of *Fusarium* spp. and the presence of *Penicillium* spp. and *Aspergillus* spp.

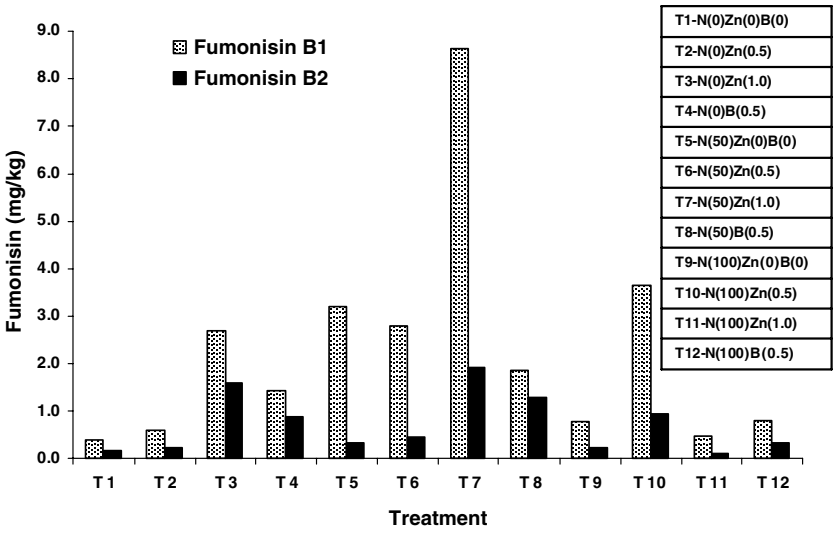

Fig. 1. Mean levels of fumonisins B_1 and B_2 in grain samples obtained from freshly harvested corn (135 days after sowing).

in air have been previously reported (Almeida et al., 2002; Gambale et al., 1983).

Mycotoxicological analysis of grain samples obtained from freshly harvested corn revealed the presence of fumonisins at levels ranging from 0.3 to 24.3 mg/kg for FB_1 and from 0.05 to 5.42 mg/kg for FB_2.

Regression analysis showed a quadratic effect of nitrogen (in combination with boron) concentration on FB_2 production ($p < 0.05$, $r^2 = 1.0$), with the production of 0.89, 1.28 and 0.35 mg FB_2/kg in treatments 4 [N(0)B(0.5)], 8 [N(50)B(0.5)] and 12 [N(100)B(0.5)], respectively (Fig. 1). Except for treatment 10 [N(100)Zn(0.5)], an increase in nitrogen concentration resulted in reduced FB_1 levels (Fig. 1). In this respect, Blandino et al. (Blandino, Alma, Matta, & Reyneri, 2004) also observed a decrease of fumonisin concentrations in corn grains with the use of increased doses or amounts of nitrogen (0, 100, 200, 300 and 400 kg/ha). In Argentina, Ramirez et al. (1997), evaluating the effects of fertilization on different plantation areas, found no effect of fertilization with urea and dibasic ammonium phosphate on fumonisin production when each area was considered separately. There was a negative linear effect of nitrogen concentration on water activity ($p < 0.01$, $r^2 = 0.87$), with an increase in nitrogen concentration leading to a reduction in the water activity of corn grains. This finding might be explained by the fact that nitrogen is responsible for an increase in the protein content of corn grains (Yamada & Abdalla, 2000). Ferreira, Araújo, Pereira, and Cardoso (2001) observed that increasing doses of nitrogen (0–210 kg/ha) applied to the corn culture increased grain protein content from 7.5% to 10.5%. In addition, binding of proteins to water also contributes to a decrease in the quantity of free water (water activity) of corn grains (Bourne, 1987). These facts probably explain the lower fumonisin levels since, according to Marin et al. (Marin, Homedes, Sanchis, Ramos, & Magan, 1999), reduced water activity levels impair the growth of *F. verticillioides*.

In Brazil, the recommended nitrogen and zinc doses to be applied during sowing to obtain the best corn crop yield range from 60 to 120 kg/ha and from 0.5 to 1.2 kg/ha, respectively (Coelho, França, Pitta, Alves, & Hernani, 2002; Fancelli & Dourado, 2000). On the other hand, boron, due to its low mobility in the plant, should be applied through soil in a corrective manner to the total area or during sowing, with the recommended amount of this element ranging from 0.2 to 0.8 kg/ha (Fancelli & Dourado, 2000). These recommended doses were also used in the present investigation.

Under the conditions of the present experiment, an increased zinc concentration resulted in an increase in the relative frequency of *F. verticillioides* and in FB_2 production, but led to a decrease in the relative frequency of *Penicillium* spp. The combination of nitrogen (100 kg/ha) with zinc (0 and 1.0 kg/ha) and boron (0 and 0.5 kg/ha) resulted in reduced FB_1 and FB_2 levels. These results indicate the need for the careful use of macronutrients and, especially, micronutrients not only to increase corn productivity but also as a control measure of natural contaminants such as fungi and mycotoxins.

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