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Research Paper

Fusarium species and fumonisins associated with maize kernels produced in Rio Grande do Sul State for the 2008/09 and 2009/10 growing seasons

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Abstract

Ear rots caused by *Fusarium* spp. are among the main fungal diseases that contribute to poor quality and the contamination of maize grains with mycotoxins. This study aimed to determine the visual incidence of fungal-damaged kernels (FDKs), the incidence of two main *Gibberella* (a teleomorph of *Fusarium*) complexes (*G. fujikuroi* and *G. zeae*) associated with maize using a seed health blotter test, and the fumonisin levels, using high performance liquid chromatography, in samples of maize grains grown across 23 municipalities during the 2008/09 and 2009/10 growing seasons. Additionally, 104 strains that were representative of all of the analysed samples were identified to species using PCR assays. The mean FDK was seven per cent, and only six of the samples had levels greater than six per cent. *Fusarium* spp. of the *G. fujikuroi* complex were present in 96% of the samples, and *G. zeae* was present in 18% of the samples (5/27). The mean incidence of *G. fujikuroi* was 58%, and the incidence of *G. zeae* varied from 2 to 6%. FB_1 was found in 58.6%, FB_2 in 37.9%, and both toxins in 37.9% of the samples. The FB_1 and FB_2 levels were below the quantification limits for 41.3% of the samples, and the mean FB_1 levels (0.66 μg/g) were higher than the mean FB_2 levels (0.42 μg/g). The PCR identification separated the 104 isolates into three of the *G. fujikuroi* complex: *F. verticillioides* (76%), *F. subglutinans* (4%) and *F. proliferatum* (2%); and *G. zeae* (anamorph = *F. graminearum*) (18%). Our results confirmed the dominance of *F. verticillioides*, similar to other regions of Brazil, but they differed due to the relatively higher incidence of *F. graminearum*. Total fumonisin levels were below the maximum limit determined by current Brazilian regulations.

Key words: *Fusarium graminearum*, *Fusarium verticillioides*, fumonisins, *Zea mays* L.

Introduction

In Brazil, maize (*Zea mays* L.) is grown under a diverse range of climate and cropping conditions. In the southernmost subtropical climate, maize is a typical summer crop, succeeding small-grain cereal crops. Several diseases potentially limit maize yields, such as those affecting leaves, stalks and ears, and are caused by several pathogenic fungi (White, 1999; Casa and Reis, 2003). Ear rots caused by various fungi, including *Fusarium* species, contribute to poor grain quality and contaminate grains with mycotoxins, which represent a threat to both human and animal health (Logrieco *et al.*, 2002; Munkvold, 2003).

Two *Fusarium*-induced ear rots are commonly found in Brazil, eventually in association: Fusarium ear rot (FER) caused by species within the *Gibberella fujikuroi* complex, especially its anamorphic species, *F. verticillioides*, *F. subglutinans* and *F. proliferatum*, and Gibberella ear rot (GER) caused by *Gibberella zeae*, the teleomorphic stage of the *Fusarium graminearum* species complex (Casa and Reis, 2003; Reis *et al.*, 2004).

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Climatic conditions and crop management practices, such as crop rotation, tillage, planting date and fertilisation, influence the occurrence and prevalence of the *Fusarium* species that affect maize (Munkvold, 2003). In some regions where both species are present, GER epidemics are most commonly found during wet years, whereas FER epidemics tend to occur during dry years (Doohan *et al.*, 2003). In Europe, *F. verticillioides* is more prevalent in the southern regions and is found associated with maize grain and by-products in France, Spain and Italy (Bottalico, 1998). In Belgium, however, *F. graminearum* is the most prevalent species associated with *Fusarium*-induced ear rots (Scauflaire *et al.*, 2011); in New Zealand, *F. graminearum* is also the most dominant species among several others found to infect kernels, whereas *F. verticillioides* is rarely found (Hussein *et al.*, 2002). In Africa, several reports indicate *F. verticillioides* as the most prevalent fungus on maize (Fandohan *et al.*, 2003).

In Brazil, empirical evidence shows that *F. verticillioides* is the most prevalent species causing FER in the central-western, tropical maize production regions (Almeida *et al.*, 2002), whereas *F. graminearum* is more commonly found in the southernmost regions of the country, although it is second in prevalence. In the southernmost region, a combination of a wet subtropical environment and a rotational system that includes small grains, such as wheat, barley and oats, favour inoculum accumulation and epidemics of GER and stalk rots caused by *F. graminearum* (Casa and Reis, 2003; Reis *et al.*, 2004).

Surveys for mycotoxins in Brazilian maize grain are sporadic, and most studies have focused on the detection of fumonisins, a group of mycotoxins produced by *F. verticillioides* and other related species of the *G. fujikuroi* complex that affects maize, such as *F. subglutinans* (Marasas, 2001). In a large spatial-scale survey and analysis of 214 maize grain samples from several locations in Brazil, 99% of the samples were contaminated with fumonisin B_1 (FB_1) in levels ranging between 0.2 and 6.1 μg/g (Vargas *et al.*, 2001). More recently, trichothecene mycotoxins, especially deoxinivalenol (DON) and nivalenol (NIV), a class of mycotoxins mainly produced by members of the *F. graminearum* species complex, have been found in 16 and two samples, respectively, out of 80 samples of Brazilian maize grains (Milanez and Valente-Soares, 2006).

Because the type of mycotoxins found in grain is dependent on the toxigenic profile of the pathogenic populations in the field, knowledge on the prevailing species and mycotoxin levels may help to develop regional strategies aimed at preventing both the ear rot and stalk rot caused by the local populations. Additionally, the promulgation of maximum tolerance levels of fumonisins in Brazilian maize grain and by-products established in 2011 reinforces the need to increase vigilance and define strategies to prevent mycotoxin contamination based on the regional status (Anvisa, 2011).

Therefore, the main objective of this study was to determine the post-harvest incidence of two *Fusarium* species complexes, the fumonisin levels and the incidence of visibly fungal-damaged maize kernels in a sample of maize grains from the major production regions of Rio Grande do Sul State, Brazil, obtained during two consecutive growing seasons across several locations. Additionally, *Fusarium* strains representative of the groups found in all of the grain samples were identified to the species level using PCR assays.

Materials and Methods

Study area and sampling

A total of 29 maize grain samples were obtained from one to two months after the harvest of maize crops in experimental areas or fields located across 23 municipalities in Rio Grande do Sul State, Brazil. The majority of the municipalities were located in the northern production regions of the state (Figure 1). Sixteen maize samples were from the 2008/09 growing season and thirteen from the 2009/10 season. The grain samples were collected by collaborators in both experimental and commercial fields, and information, such as the hybrid and cropping practices, were not available for most of the samples.

Incidence of Fusarium and visibly fungal-damaged kernels

A subsample of 100 kernels, randomly taken from a 500 g field grain sample, was assessed in the laboratory for symptoms and signs of fungi infection, especially shrivelled and discoloured kernels. Each kernel was visually inspected to determine whether it had any symptom or sign of fungal infection. The incidence of visibly fungal-damaged kernels was expressed as a percentage.

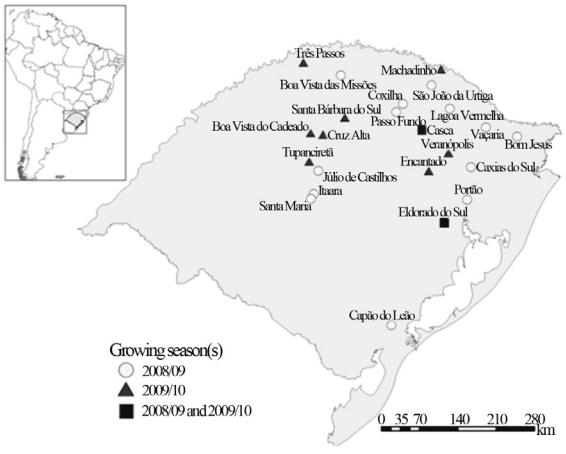

Figure 1 - Map depicting the location of 23 municipalities in Rio Grande do Sul State, Brazil, where 29 maize kernel samples were harvested in one and/or two maize growing seasons, 2008/09 and 2009/10.

The incidence of *Fusarium* infection was determined using an adapted protocol of a standard freezing-blotter seed health test (Machado and Langerak, 2002). A subsample of 200 kernels was surface sterilised (1% sodium hypochlorite for 1 min) and rinsed twice in sterile, distilled water (30 seconds). The kernels were plated (25 per recipient) equidistantly on moist blotters and incubated at 25 °C for 10 days. Thereafter, each kernel was inspected with the aid of a stereomicroscope (40x magnification), and the percentage of kernels showing colonies resembling species belonging to either the *Gibberella fujikuroi* or *Gibberella zeae* species complex was determined. All of the ratings were performed by an experienced rater, and, whenever needed, slides of the microscopic structures were prepared to aid the classification to one of the two species complexes.

Determination of fumonisins by high-performance liquid chromatography (HPLC)

Fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) were determined using an established protocol (Camargos *et al.*, 1999), with modifications. The fumonisin standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The working standard solutions were prepared in acetonitrile-water (1:1), with concentrations of 5 μg/mL each for FB_1 and FB_2. An aliquot of each sample (50 g) was extracted with 100 mL of methanol-water (3:1) for 5 min in a blender (Waring Co., Torrington, USA). After centrifugation and filtering, the pH of the filtrate was adjusted to 5.8-6.5 with 0.1 N HCl or 0.1 N NaOH. A strong anion exchange cartridge (500 mg, Sep-PaK, Waters), was used to purify the sample, previously conditioned with 6 mL of methanol (Honeywell Burdick & Jackson, Muskegon, USA) and 10 mL of methanol-water (3:1). An aliquot (10 mL) of the sample extract was applied to the cartridge, followed by 10 mL of methanol-water (3:1) and 6 mL of methanol. The FB_1 and FB_2 peaks were eluted with 20 mL of methanol-acetic acid (95:5). The eluate was dried in a sample concentrator at 60 °C (Tecnal, Piracicaba, Brazil) and kept at -20 °C until it was analysed. The dried extract was dissolved in 1000 μL of acetonitrile-water (1:1). The chromatographic system used was the Finnigan Surveyor Plus model (Thermo Scientific®, San Jose, USA), and post-column derivatisation was performed by Vector PCX (Pickering Laboratories®, Mountain View, USA) with fluorescence detection using a Finnigan Surveyor FL Plus Detector (Thermo Scientific®, San Jose, USA). The eluate (100 μL) was injected in the chromatograph under the following conditions: a mobile phase of methanol-sodium phosphate buffer, pH 3.35 (70:30); a flow rate of 0.5 $mL.min^{-1}$; an excitation wavelength of 330 nm and an emission at 465 nm; a C18 Spherisorb® column of 5 μm (250 x 4.6 mm, Waters®, Wexford, Ireland); a total run time of 30 min; and integration by ChromQuest 5.0 Chromatography Data System (Thermo Fisher Scientific®). The post-column reagent pump flow rate was set at 0.15 mL/min and the reactor temperature at 65 °C. The OPA reagent (Pickering Laboratories®, Mountain View, USA) was used for the derivatisation of the fumonisins. The average recovery and the variation coefficients for FB_1 and FB_2 were 84.6% and 8.1% and 102.9% and 6.2%, respectively. The limit of quantification was 0.078 and 0.043 μg/g for FB_1 and FB_2, respectively.

Fungal isolation, purification and DNA extraction

During the assessments of the seed health test, four *Fusarium* isolates from each of the 29 samples were obtained. Each isolate was randomly selected from an individual recipient, and the selection of the colony accounted for the morphological differences, such that the four isolates could represent potentially different groups at the same proportion found in each grain sample. A fragment of mycelia from the selected *Fusarium* colony was transferred to malt agar media (2%) and incubated in a growth chamber (24 ± 1 °C under dark conditions) for seven days. The isolates were purified through monosporic culturing, transferred to SNA medium (Leslie and Summerell, 2006) and stored in microtubes (1.5 mL) under cold conditions (4 °C). Mycelia were produced in liquid potato-dextrose media amended with 50 mg/L of streptomycin sulphate (Sigma-Aldrich®) under shaking conditions for ten days and at ± 25 °C. The DNA was extracted using a modified 2% CTAB (Hexadecyl trimethyl-ammonium bromide) DNA extraction protocol (Doyle and Doyle, 1987) and was stored at -20 °C until analysis.

PCR-based species identification

All of the selected and purified isolates were identified to the species level using polymerase chain reaction (PCR) assays found in the literature using primer sets (VER1, VER2, PRO1, PRO2, SUB1 AND SUB2) for the differentiation of three species of the *G. fujikuroi* complex (*F. verticillioides*, *F. proliferatum* and *F. subglutinans*) (Mulé *et al.*, 2004) and a primer set for *Gibberella zeae* (*Fusarium graminearum* species complex), Fg16F/R (Nicholson *et al.*, 1998). The PCR assays were conducted using 20-30 ng of fungal DNA in a total volume of 25 μL containing 1.5 mM $MgCl_2$, 2 U Taq DNA polymerase, 20 μM dNTPs, and 1 μM of each primer. The PCR products were separated by gel electrophoresis, stained with SYBR® Safe DNA gel and visualised under UV light.

Results and Discussion

Visibly fungal-damaged kernels (FDKs) were found in 16 out of the 29 samples. The mean FDK incidence was seven per cent, and six samples had levels greater than six per cent, which is considered the maximum tolerated level for the corn trade in Brazil (Pinto *et al.*, 2007); three samples had incidence levels > 25%. This result parallels previ-

ous reports in the country in which the differences in the FDK levels were attributed to the cultivars or incidence of fungal species other than *Fusarium* (Pinto *et al.*, 2007).

All but one (Santa Maria, 2009/10) of the maize samples was infected with one or two species of the *Gibberella* complexes (Table 1). The two species complexes co-occurred in the samples, but a distinct frequency of these two complexes was found: *G. fujikuroi* was present in 96% of the samples and *G. zeae* in 18% (5/27 samples). Overall, the mean incidence of *G. fujikuroi* was 58% (51% in 2008/09 and 67% in 2009/10), and the mean incidence of *G. zeae* varied from 2 to 6% and was mostly limited to samples from the 2008/09 growing season (Table 2). With regards to the presence of fumonisins, 58.6% of the samples were contaminated with FB_1, 37.9% with FB_2, and 37.9% with both fumonisins (Table 1). The FB_1 and FB_2 levels were below the detection limit for 41.3% of the samples. The overall mean FB_1 level (0.66 μg/g) was higher than the mean FB_2 levels (0.42 μg/g).

Compared to previous studies, the fumonisin levels were, for the majority of the samples, lower than those reported in 109 maize grain samples from the state of Paraná, Brazil (Ono *et al.*, 2006). In that study, FB_1 and FB_2 were

Table 1 - Data on visibly fungal damage kernels (FDK), *Gibberella fujikuroi* species complex (GF), *Gibberella zeae* (anamorph = *Fusarium graminearum sensu lato*) (GZ) and fumonisin levels (FB_1 and FB_2) associated with maize samples collected across 23 municipalities in Rio Grande do Sul State, Brazil, 2008/09 and 2009/10 growing seasons.

Year	Location	FDK (%)	GF (%)	GZ (%)	FB_1 (μg/g)	FB_2 (μg/g)
2008/09	Boa Vista das Missões	13	36	0	1.04	0.81
	Boa Vista das Missões	4	39	0	1.63	0.71
	Casca	0	58	0	0.29	0.33
	Caxias do Sul	0	44	0	0.23	ND
	Coxilha	61	39	3	1.18	ND
	Júlio de Castilhos	5	29	0	ND	ND
	Lagoa Vermelha	0	73	0	ND	ND
	Passo Fundo	5	71	2	ND	ND
	Passo Fundo	0	9	0	0.73	ND
	Portão	3	64	6	0.30	0.18
	Santa Maria	29	0	0	ND	ND
	São João da Urtiga	1	52	0	ND	ND
	Vacaria	7	69	0	ND	ND
	Vacaria	4	87	0	ND	ND
	Vacaria	0	96	4	ND	ND
	Vacaria	0	NA	NA	ND	ND
Year mean		8.25	51.0	1	0.77	0.51
2009/10	Boa Vista do Cadeado	0	61	0	ND	ND
	Boa Vista do Cadeado	0	100	0	ND	ND
	Casca	15	79	4	0.75	0.16
	Casca	0	NA	NA	0.35	0.15
	Cruz Alta	2	66	0	ND	ND
	Eldorado do Sul	4	94	0	0.10	ND
	Encantado	4	78	0	2.03	0.81
	Encantado	0	75	0	0.32	0.27
	Machadinho	0	28	0	0.60	0.13
	Santa Bárbara	41	70	0	0.29	0.21
	Três Passos	5	37	0	1.01	0.84
	Tupanciretã	0	20	0	0.07	ND
	Veranópolis	0	97	0	0.09	ND
Year mean		5.46	67.08	0.33	0.56	0.37
Total mean		7	58.1	0.70	0.66	0.42

NA = Data not available; ND = Values lower than the quantification limit (FB_1: -0.078 $\mu g.g^{-1}$ e FB_2: -0.043 $\mu g.g^{-1}$).

Table 2 - Number (and total %) of isolates for each *Fusarium* species and growing season, determined using PCR assays, associated with samples of maize kernels from 23 municipalities in Rio Grande do Sul State, Brazil, 2008/09 and 2009/10 growing seasons.

Fusarium species	2008/09	2009/10	Total
Fusarium verticillioides	41	38	79 (75%)
Fusarium subglutinans	4	0	4 (4%)
Fusarium proliferatum	0	2	2 (2%)
Fusarium graminearum sensu lato	15	4	19 (19%)
Total	60 (57%)	44 (43%)	104 (100%)

determined separately for symptomatic and asymptomatic kernels, and the mycotoxin levels varied between 0.57 to 20.38 μg/g for the asymptomatic kernels and from 68.98 to 336.38 μg/g for the symptomatic kernels. In our study, FB_1 and FB_2 were also detected in asymptomatic samples, which is also in agreement with a previous report (Ottoni, 2008) in which relatively low fumonisin levels were found, varying from non-detected to 2.1 μg/g in 15 asymptomatic maize grain samples from the Brazilian states of Minas Gerais, Goiás, Mato Grosso, Mato Grosso do Sul and Paraná.

In our study, the three samples showing extreme FDK values did not show the highest fungal incidence or fumonisin levels. Conversely, a relatively high incidence of *Fusarium* species of the *G. fujikuroi* complex was found in the thirteen samples with 100% of the asymptomatic kernels. In fact, it is well know that *F. verticillioides* is able to colonise the plant systemically and invade grains without causing symptoms, which can explain the lack of correlation between symptoms and *Fusarium* incidence or mycotoxin levels (Munkvold, 2003).

Differences in the mycotoxin levels across studies are expected, even for the same region, as they are under the influence of many biological, abiotic and agronomic factors, such as the toxigenic potential of the prevailing fungal species, host genetics, management practices and environmental conditions (Munkvold, 2003). Hence, continuous surveillance is appropriate, especially when linking analytical and epidemiological survey data aiming to understand risk factors related to mycotoxin contamination. In our study, the dominance of *F. verticillioides* over the other species confirms previous survey studies with maize grain from other regions of Brazil (Ono *et al.*, 1999; Buiate *et al.*, 2008). The presence of *F. graminearum sensu lato* at relatively lower prevalence and incidence levels in maize kernels is in agreement with a previous study in the Rio Grande do Sul State, where it was found to be the second species in prevalence in a monoculture *vs.* crop rotation study (Trento *et al.*, 2011). Collectively, these results suggest that maize kernels from the southernmost region of Brazil may also be contaminated with trichothecene mycotoxins because of the presence of *F. graminearum sensu lato*, as has been previously shown in a few maize samples from other Brazilian regions (Milanez and Valente-Soares, 2006).

Of the 104 monosporic isolates obtained from grains representative of all of the maize samples, the PCR-based identification showed the presence of four anamorphic species of the two *Gibberella* complexes: *F. verticillioides* was the dominant species (76% of the isolates), followed by *F. graminearum* (18%), *F. subglutinans* (4%) and *F. proliferatum* (2%) (Table 2). Molecular tools have been used to identify *Fusarium* species from maize in Brazil only very recently (Ottoni, 2008; Querales, 2010), and their use can be advantageous given the accuracy of the results, especially for the differentiation of species of the G. *fujikuroi*, which demands several steps and experience in the identification based on morphological traits. Our findings of the three anamorphic species within the *G. fujikuroi* complex, especially the presence of *F. proliferatum* and *F. subglutinans* in relatively low prevalence (< 5%), is in agreement with previous studies conducted in Brazil using PCR assays. Ottoni (2008) has analysed 197 isolates from maize grains obtained in several growing regions of Brazil and demonstrated the prevalence of *F. verticillioides* (82%) and *F. subglutinans* (3%); none of the isolates were identified as *F. proliferatum*. Moreover, using the same protocol as Ottoni (2008), Querales (2010) analysed 100 isolates from maize kernels produced in the states of São Paulo, Minas Gerais, Bahia, Paraná, Rio Grande do Sul and Mato Grosso do Sul during 2001 to 2006 and identified *F. verticillioides* (93% of the isolates), *F. proliferatum* (4%) and *F. subglutinans* (3%).

Our results add to the current knowledge and confirm the dominance of *F. verticillioides* within the species of the *G. fujikuroi* complex, across the production regions of Rio Grande do Sul State, in prevalence levels similar to those observed in other regions of Brazil. Additionally, we found *F. graminearum sensu lato* to be the second in prevalence in the PCR-based identification. All of the isolates taken from the *G. zeae*-like colonies were identified as *F. graminearum sensu lato* using the Fg16F/R primer set, which demonstrated that the separation of the two *Gibberella/Fusarium* complexes based on morphology was accurate during the evaluation of the seed health test by an experienced rater. Conversely, the distinction of the three *Fusarium* species of the *G. fujikuroi* complex was not accurate based on the colony morphology, when compared to the results of the PCR assays (data not shown).

As found in this study, the higher prevalence of *G. zeae* in Southern Brazil compared to other regions of Brazil may be due to cooler climatic conditions and the year-round presence of inocula from epidemics caused by *F. graminearum* in small-grained cereals, mainly wheat and barley, and the survival of the sexual stage of the fungus (perithecia) in crop residues. This pattern parallels the situation found in Argentina, where species of the *G. fujikuroi*, mainly *F. verticillioides*, and *F. graminearum*

sensu lato, have been frequently isolated from maize kernels, especially in the northwestern region of that country for the latter species (Chulze *et al.*, 1996; Sampietro *et al.*, 2011).

A higher diversity of *Fusarium* species was found associated with maize kernels produced in the southernmost maize-growing regions of Brazil compared to previous reports from other northern regions. Thus, a range of *Fusarium* mycotoxins other than fumonisins can be produced by the regional populations, especially the trichothecenes, deoxynivalenol and nivalenol, which are produced by the *F. graminearum sensu lato* populations that affect wheat and barley grown in the same region (Scoz *et al.*, 2009; Astolfi *et al.*, 2011). The fumonisins in our study were found at levels that are considered safe for consumption because none presented a level greater than the maximum limit ($FB_1 + FB_2$) of 5 μg/g determined by the current Brazilian regulation for mycotoxins (Anvisa, 2011). Future studies shall be directed at elucidating the toxigenic potential of the representatives of these populations and the analysis of the presence of other *Fusarium* mycotoxins as an important step towards the definition of strategies to prevent and minimise the risk of maize contamination in Southern Brazil.

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