

Genetic structure of *Fusarium verticillioides* populations and occurrence of fumonisins in maize grown in Southern Brazil



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ABSTRACT

The fumonisins are a group of mycotoxins produced primarily by *Fusarium* spp. There are several different forms of fumonisins, among them fumonisins B₁ and B₂ are the most common and economically important forms in maize. The aim of this study was to investigate the presence of fumonisins and *Fusarium* spp. in kernels of four maize genotypes grown in two Southern Brazilian locations. Fumonisins B₁ and B₂ were detected in all samples, with levels ranging from 0.4 to 9.1 µg × g⁻¹. Of the 3840 maize kernels examined, 77.0% were infected with *Fusarium* spp., and *F. verticillioides* was the most prevalent species (98.1%). In addition, we found that approximately 95% of the isolates of *F. verticillioides* harbor essential genes for fumonisin biosynthesis (*FUM1* and *FUM8*). Next, we investigated the genetic structure of *F. verticillioides* populations based on molecular data generated by the AFLP technique, which revealed a high genetic variability. Statistical analyses have shown that a significant part of the genetic differentiation was associated with the maize growing location.

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1. Introduction

Brazil has a land area of 8,511,996 square kilometers, making it the largest country in South America and the fifth largest country in the world. Maize is planted in all regions of the country, and is cultivated under a diverse range of climate and cropping conditions. The Brazilian production of maize is around 80 million tons of grains per year, ranking Brazil as one of the world's largest producers and exporters of this commodity (FAOSTAT, 2017). Although this crop definitely plays a very significant role in the country's economy, contaminations of maize kernels with mycotoxins, especially those from *Fusarium* spp., are still a significant problem and a food safety challenge to be overcome.

Fusarium spp. and mycotoxins incidence in maize kernels can be influenced by various factors, including climatic conditions, availability of water, chemical composition of the grain, plant-pathogen interaction, genetic factors intrinsic to the genotype, and farming practices (Arias et al., 2012; Blandino et al., 2009; Cao et al., 2014; Chatterjee et al., 2016; Dall'Asta et al., 2012; Marin et al., 2013;

Rocha et al., 2016; Santiago et al., 2015; Waskiewicz et al., 2013). Regarding to farming practices, dose and the type of N fertilizer application can influence the incidence and mycotoxins contamination in maize kernels (Abbas et al., 2009; Blandino et al., 2008). Although not with the specific objective of mycotoxin control, several researchers have attempted to perfect the technology for inoculating grasses, including maize, with plant growth-promoting bacteria (PGPB) (Berta et al., 2014; Dhawi et al., 2015; Hungria et al., 2010; Islam et al., 2016). These bacteria benefit plant growth by combining various mechanisms, such as the enhanced nitrogen biological fixation capacity and the activity as pathogen biological control agents (Hungria et al., 2013; Pérez-Montaña et al., 2014). Nevertheless, there is no information on the effects of PGPB inoculants on the incidence of *Fusarium* spp. and fumonisin in field maize.

Fumonisins are the most frequently detected mycotoxins in maize kernels. They are a group of polyketide-derived mycotoxins, which cause leukoencephalomalacia in horses (Marasas et al., 1988), pulmonary edema in pigs (Haschek et al., 2001) and probably esophageal cancer in humans (Yoshizawa et al., 1994). There are at least 28 different forms of fumonisins (Alberts et al., 2016), but fumonisins B₁ (FB₁) and B₂ (FB₂) have been considered the most economically important forms (Alizadeh et al., 2012;

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Falavigna et al., 2012; Streit et al., 2012). The occurrence of FB₁ and FB₂ in maize and maize-based products in Brazil has been reported by several authors (Lanza et al., 2014; Scussel et al., 2013; Stumpf et al., 2013).

The type of mycotoxins found in maize kernels is dependent on the toxicogenic profile of the pathogenic populations in the field. The knowledge on the prevailing *Fusarium* species is important to help the development of regional strategies aimed at preventing mycotoxin contamination (Stumpf et al., 2013). The main fungal species responsible for the production of fumonisins are *Fusarium verticillioides* (Sacc) Nirenberg and *Fusarium proliferatum* (Matsushima) Nirenberg, both frequently found in maize kernels (Bryla et al., 2013; Zhang et al., 2012). The predominant of one or another fungal species can vary according to the geographic region and environmental conditions (Ferrigo et al., 2016).

Over the last 10 years, molecular markers have been used to precisely identify *Fusarium* species and to assess genetic diversity of some species of this genus (Astolfi et al., 2011; Castañares et al., 2016; Lee et al., 2009; Momeni and Nazari, 2016; Olowe et al., 2017; Reynoso et al., 2009; Rocha et al., 2011; Wang et al., 2010). Amplified fragment length polymorphism, for example, was used to provide information about the genetic structure of *Fusarium verticillioides* populations isolated from maize around the world (Covarelli et al., 2012; Reynoso et al., 2009; Tsehay et al., 2016). However, only a very few studies using molecular markers were carried out to provide information on the amount and the distribution of genetic variation within and among Brazilian *Fusarium* populations (Astolfi et al., 2011).

In this scenario, the aim of this study was to investigate the presence of fumonisins and *Fusarium* spp. in maize kernels of four maize genotypes grown in two Southern Brazilian locations, under different nitrogen fertilization conditions, including the use of PGPB. Additionally, we investigated the genetic structure of *F. verticillioides* populations and if genetic differentiation was associated with maize growing locations.

2. Materials and methods

2.1. Field trial and sampling

The experiments were conducted in November 2012 in Londrina (23° 18' 36" S, 51° 09' 46" W) and Florestópolis (22° 51' 48" S, 51° 23' 14" W), both locations are in the State of Paraná, one of the leading maize production regions in Brazil. Temperature and precipitation data were retrieved from The Instituto Agronômico do Paraná, Brazil in regard to both locations (Fig. S1).

In order to evaluate four maize genotypes and different kinds of nitrogen fertilization, the experiment was carried out in a randomized block design with four replications in a 4 × 4 factorial arrangement. Each plot consisted of six rows 4 m long, spaced 0.9 m apart, with individual plants spaced 0.2 m. Only the four middle rows were used for analyzing the incidence of infection by *Fusarium* and fumonisin contamination.

The following genotypes were evaluated: a) a commercial hybrid; b) variety ST0509 (V1); c) variety ST1309 (V2); and d) a landrace denoted "Caiano" (CA). The ST0509 and ST1309 varieties were synthesized from the Carioca and the Caiano landraces, respectively, which were pollinated by nine elite inbred lines developed by the maize breeding program of the Universidade Estadual de Londrina, Brazil. These elite inbred lines have modern plant architecture, earliness, yellow endosperm, and potential for the synthesis of maize hybrids with high yield performance. On the other hand, the landrace variety has yellow endosperm, late flowering, older architecture, larger leaves, taller plants, lower stalk lodging resistance, greater rusticity and tolerance to environmental

stress. They are cultivated and maintained by family farmers of Paraná State, Brazil, using low-level input and agricultural mechanization.

Four kinds of fertilization were evaluated: a) single application of nitrogen at planting (SA); b) complete fertilization involving nitrogen application at planting and top dressing, with no inoculation (TD); c) nitrogen application at planting combined with a commercial inoculant Ab-V5, registered with the Brazilian Ministry of Agriculture, Fisheries and Supply, containing a strain of *Azospirillum brasilense* (BV); d) nitrogen application at planting and inoculant ZM containing a strain of *Methylobacterium komagatae* isolated from sunflower (ZM) (Goes et al., 2012). Mineral fertilizer was applied at a rate of 350 kg ha⁻¹ [4-14-8 NPK (14 kg nitrogen + 49 kg phosphorus + 28 kg potassium)]. The top-dressed mineral fertilizer was applied at a rate of 100 kg ha⁻¹ nitrogen.

After kernels approached physiological maturity, when the moisture level reached about 16%, dried maize cobs were harvested separately by rows of each plot. A mixture of kernels (200 g) was sampled from each row, totaling 800 g per plot. These samples were stored in paper bags properly identified and used for analysis of *Fusarium* infection and fumonisin contamination.

2.2. Seed inoculation with plant growth-promoting bacteria

The bacterial strains were grown in 250 mL of M15 liquid medium (composition patent pending) for 48 h at 28 °C, stirred at 130 rpm. The cell concentration was estimated by spectrophotometry at 560 nm. After normalizing the suspension at 2.5 × 10⁹ cells mL⁻¹, the liquid inoculant was prepared (composition patent pending). The final cell concentration in the inoculant was 10⁹ cells mL⁻¹. A volume of 20 mL of each inoculant (Ab-V5 or ZM) was mixed with 1 kg maize seeds 12 h before planting.

2.3. Quantification of fumonisins B₁ and B₂

For fumonisin quantification, 200 g of maize kernels were taken from each sample. To fumonisin extraction, 10 g of homogenized ground samples of maize kernels were added to 30 mL methanol: water (3:1, v/v), stirred at 150 rpm for 30 min and then filtered using Whatman No. 1 filter paper. The filtrate (1 mL) was cleaned up in a Sep-Pak Accell Plus QMA anion exchange cartridge (Waters, USA), previously conditioned with 5 mL methanol followed by 5 mL methanol: water (3:1, v/v). After washing the cartridge with methanol: water (3:1, v/v, 6 mL) followed by methanol (3 mL), the fumonisins were eluted with 10 mL 0.5% acetic acid in methanol. The eluent was dried at 45 °C and the residue resuspended in 800 μL methanol: water (3:1, v/v). Aliquots of 200 μL were then, dried under an N₂ gas stream at 45 °C, and frozen (-20 °C) for subsequent fumonisin analysis. Samples were resuspended in 50 μL acetonitrile: water (1:1, v/v) and after derivatization with 200 μL O-phthalodialdehyde (OPA; Sigma, USA) solution (40 mg OPA, 1 mL methanol, 5 mL 0.1 M sodium borate and 50 μL 2-mercaptoethanol), injections were made within 1 min. In each application 20 μL of the reaction product were injected into the High Performance Liquid Chromatography (HPLC). Fumonisins (FB₁ and FB₂) were quantified by a reversed-phase isocratic HPLC according to Shephard et al. (1990) method modified by Ueno et al. (1993). The HPLC system consisted of an LC-10 AD pump, RF-10A XL fluorescence detector (Shimadzu, Japan) and C18 Luna 5 μ 100 Å column (4.6 × 250 mm) (Phenomenex, USA). Excitation and emission wavelengths were 335 and 450 nm, respectively. The eluent was CH₃OH: 0.1M NaH₂PO₄ (J.T. Baker, USA; 80: 20, v/v) adjusted to pH 3.3 with ortho-phosphoric acid (J.T. Baker). The flow-rate was 1 mL per min and the column oven temperature was set at 25 °C.

Fumonisins were quantified by comparing peak areas of

samples to a standard curve generated from FB₁ and FB₂ standard solutions (Sigma-Aldrich, USA). The limits of detection (LOD) for FB₁ and FB₂ were 27.5 ng × g⁻¹ and 35.3 ng × g⁻¹ respectively, defined as the minimum amount of toxin that could generate a chromatographic peak three times above the height/noise rate of the baseline. The limits of quantification (LOQ) for FB₁ and FB₂ were 45.8 ng × g⁻¹ and 58.8 ng × g⁻¹, defined as the minimum amount of toxin that could generate a chromatographic peak five times above the height/noise rate of the baseline. Recoveries of FB₁ and FB₂ from spiked kernel in the range 100–400 8 ng × g⁻¹ for FB₁ and 250–450 8 ng × g⁻¹ for FB₂ averaged 95.6% (mean CV 8%) and 96.9% (mean CV 10%) respectively, based on duplicate spiking and triplicate analyses.

2.4. Fungal occurrence

To evaluate the fungal incidence, 120 maize kernels from each treatment were plated directly. The maize kernels were surface disinfested with 0.4% sodium hypochlorite (NaOCl) for 2 min, rinsed twice in distilled water and dried with sterile paper. Kernels (3,840) were plated (10 grains/plate) on Dichloran 18% Glycerol agar (DG18) (Hocking and Pitt, 1980), then plates were incubated for 7 day at 25 °C, and the percentage of infected kernels was determined according to Pitt and Hocking (2009).

2.5. DNA extraction

A total of 160 isolates of *Fusarium* spp. (10 from each treatment, 80 from each location) were randomly selected and grown in Dichloran Chloramphenicol Peptone Agar (DCPA) (Andrews and Pitt, 1986) until pure colonies were obtained.

Genomic DNA was extracted using the Biopuri mini spin genomic DNA extraction kit® (Biometrix, Brazil) following the manufacturer's recommended protocol.

2.6. Protocol and statistical treatment of AFLP data

AFLP markers were amplified based on the protocol described by Vos et al. (1995), with some modifications. Briefly, genomic DNA (250 ng) from each *Fusarium* isolate ($n = 160$) was double digested with EcoRI and MseI enzymes, according to manufacturer's instructions. The DNA fragments were ligated to EcoRI and MseI-adapters using T4 DNA ligase (Thermo Fisher Scientific Inc., USA). The ligation products were submitted to pre-selective amplifications using primers that complement the EcoRI and MseI adaptors and one additional nucleotide (EcoRI+A/MseI+C). Pre-selected PCR products were used as DNA template for the selective amplification using primers with two or three additional selective nucleotides at the 3' end. Three primer combinations of 5'-fluoro-labeled EcoRI primers and unlabelled MseI primers were used: FAM-EcoRI-AAT/MseI-CA, HEX-EcoRI-AAC/MseI-CA, NED-EcoRI-AGC/MseI-CA. One μ L of the amplification product was added to a loading buffer containing 8.75 μ L Hi-Di formamide (Applied Biosystems, USA) and 0.25 μ L of GeneScan 500 ROX size standard. After denaturation, the reaction was subjected to capillary electrophoresis on an ABI3500XL® Genetic Analyzer (Applied Biosystems, USA).

GeneMapper v1.3 (Applied Biosystems, USA) was used to derive and visualize the fragment lengths of the labeled DNA-fragments, using the known fragment lengths of the ROX-labeled marker peaks. A dendrogram, based on the Jaccard similarity index was built using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

AFLP data were also used to investigate the genetic differentiation among *F. verticillioides* isolates ($n = 157$) according to their geographic origin and maize genotypes.

The 157 isolates of *F. verticillioides* were probabilistically assigned to a genetically distinct cluster based on Bayesian models using STRUCTURE 2.3.4 (Pritchard et al., 2000) program. The number of clusters (K) was set from one to eight groups, based on the number of maize genotypes ($n = 4$) and crop locations ($n = 2$). To interpret the genetic structures of the populations and their subpopulations (four from Londrina and four from Florestópolis), the ΔK (Evanno et al., 2005) statistics built into the Structure Harvester (Earl and vonHoldt, 2012) virtual platform were used.

Next, FAMD software (Schluter and Harris, 2006) was used to perform principal coordinate analysis (PCoA), in which the isolates are represented by dots in the Cartesian plane.

The allele frequencies of the set of *F. verticillioides* isolates were estimated and subjected to Analysis of Molecular Variance (AMOVA) using the Arlequin 3.5.2.2 software package (Excoffier et al., 1992). This analysis allowed partitioning genetic variability into hierarchical components (within populations and among populations) and measures the genetic differentiation by F statistic analogs (Cockerham, 1969, 1973; Excoffier et al., 1992; Weir and Cockerham, 1984).

2.7. Molecular identification of *Fusarium* spp.

For species identification, a set of 67 isolates chosen to represent different AFLP groups and subgroups was subjected to partial sequencing of the gene encoding for the translation elongation factor (*TEF-1* α). The PCR amplification was performed using the EF-1 and EF-2 primers, described in O'Donnell et al. (1998) according to the protocol described in Watanabe et al. (2011). The amplification products were purified using the Wizard SV Gel Kit and PCR Clean-Up System® (Promega, USA), according to the manufacturer's recommended protocol. The purified amplicons were sequenced using the BigDye Terminator kit, V 3.1, Cycle Sequencing® (Applied Biosystems, Austin, USA), on a ABI3500XL® Genetic Analyzer (Applied Biosystems, USA).

All *TEF-1* α sequences obtained ($n = 67$) were compared using the Basic Local Alignment Search Tool (Altschul et al., 1990) against the GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and *Fusarium* MLST databases (<http://www.cbs.knaw.nl/fusarium/>). In addition, the sequences were aligned by ClustalW algorithm with those from *Fusarium sambucinum* Species Complex and *Gibberella fujikuroi* Species Complex, obtained in the databases mentioned above, and a neighbor-joining (NJ) tree was reconstructed. For both, the MEGA 6.0 package was used (Tamura et al., 2013). To determine the support for each clade, a nonparametric bootstrap analysis was performed with 1000 resamplings.

2.8. Detection of *FUM1* and *FUM8* genes

Fusarium verticillioides ($n = 157$) were subjected to a multiplex PCR using specific primers for *FUM1* and *FUM8* genes, both essential for the synthesis of fumonisins. The primer pairs and the PCR protocol used were those described in Zhang et al. (2012) and Wang et al. (2010), respectively. The PCR products were subjected to 1% agarose gel electrophoresis. The DNA Ladder 1 kb Plus® (Invitrogen Life Technologies, USA) was used as the standard of molecular mass. Negative samples were subject to external reaction control (*TEF-1* α gene PCR) in which the DNA was assessed for amplification capacity.

3. Results and discussion

3.1. Quantification of fumonisins and *Fusarium* spp. in maize kernels

Fumonisins B₁ and B₂ were detected in all treatments at both maize growing locations (Fig. 1) at levels ranging from 0.4 to 9.1 µg g⁻¹. The concentration of fumonisins differed statistically ($p < 0.01$) between the growing locations and maize genotypes. Samples grown in Florestópolis were more contaminated (2.6–9.1 µg g⁻¹, mean of 5.0 µg g⁻¹) than those grown in Londrina (0.4–4.0 µg g⁻¹, mean of 1.8 µg g⁻¹) (Table 1), and this is not surprising as mycotoxin levels depend on the climatic conditions where fungal host samples were cultivated (Munkvold, 2003). According to Ono et al. (1999), high concentrations of fumonisins were found in regions where intense rainfall occurred in the month prior to harvesting. Similarly, the highest level of fumonisin contamination in samples from the Florestópolis site was possibly associated with the incidence of rainfalls, especially during the week prior to harvesting (Fig. S1).

The statistical analysis has shown that kernels from V1 and V2 varieties and Caiano landrace exhibited levels of fumonisins significantly lower than those of the commercial hybrid (Table 1).

The analysis for the presence of *Fusarium* spp. in 3840 maize kernels revealed that 77.0% of them were infected, and no differences were detected in the levels of infected kernels between Londrina and Florestópolis. Consequently, the higher concentration of fumonisins in kernels from Florestópolis cannot be explained by differences in the levels of fungal infection. The incidence of *Fusarium* spp. in kernels found in our study is similar to that found in other experiments conducted in Brazil and some others countries (Cao et al., 2013; Fandohan et al., 2005; Ono et al., 1999; Shala-Mayrhofer et al., 2013). Other fungi genera, such as *Aspergillus* and *Penicillium*, were also found herein but at very low frequencies (data not shown).

The results of the statistical analysis revealed that maize genotypes differed statistically ($p < 0.01$) in regard to the percentage of infected kernels by *Fusarium* spp., which was lower in the varieties (75.52% in V1 and 71.04% in V2) and in Caiano landrace (75.31%) than in the commercial hybrid (84.37%) (Table 1).

The mechanisms whereby undamaged plants and not grown from infected seeds become infected by *Fusarium* spp. have not yet been fully elucidated (Maiorano et al., 2009). Differences in the chemical composition (Dall'Asta et al., 2012) and structure of the stylar canal (Duncan and Howard, 2010) in maize genotypes have been reported as the main causes of the variation in susceptibility

to infection by *Fusarium*.

Some authors (Blandino et al., 2008; Hassegawa et al., 2008) have demonstrated the influence of fertilization on contamination with fumonisins, but in our study no significant difference was found between the fertilization treatments for both, fumonisin concentration and incidence of kernels infected by *Fusarium* (Table 1). This study was the first to investigate the effect of PGPB inoculants on the incidence of *Fusarium* spp. and fumonisin in maize and no association was found.

3.2. Molecular identification of *Fusarium* spp. isolates

A total of 160 isolates of *Fusarium* spp. were subjected to AFLP analysis using three enzyme-primer combinations that generated 757 loci ranging from 50 to 500 bp in size. UPGMA grouping analysis revealed the formation of four main groups (GI, GII, GIII and GIV) with a level of 50% genetic similarity. GI contained the majority of fungal isolates (Fig. S2). To species identification, a set of 67 isolates chosen to represent all AFLP groups and subgroups was subjected to partial sequencing of the *TEF-1α* gene. These nucleotide sequences were used to infer a phylogenetic tree (Fig. 2), together with the sequences of *Fusarium* strains available in GenBank and *Fusarium* MLST. Among the 67 isolates, a total of 64, all belonging to GI, were identified as *F. verticillioides*, thus confirming the dominance of this species in Brazilian maize kernels.

In addition to *F. verticillioides*, we found only one isolate of the following species, *Fusarium subglutinans* (GII), *F. graminearum* (GIII), and *F. proliferatum* (GIV). *F. subglutinans* has been described as one of the most frequent species in asymptomatic maize, with high toxicity associated with the production of beauvericin and moniliformin (Deepa et al., 2016; Desjardins et al., 2006; Moretti et al., 2008). *F. graminearum* is one of the species commonly found in cereal grains and capable to produce the deoxynivalenol and zearalenone toxins (Häggblom and Nordkvist, 2015). *F. proliferatum*, like *F. verticillioides*, has been frequently detected in maize and considered one of the main producers of fumonisins in cereals (Jurado et al., 2010; Zhang et al., 2012).

3.3. Genetic structure of *Fusarium verticillioides* population

Based on 709 AFLP loci detected in 157 *F. verticillioides* isolates, the fungal population structure was analyzed using three statistical treatments as mentioned in the Material and methods section. First, an attribution test has shown that the ideal cluster number for allocating all the isolates was two ($K = 2$). The clustering results separated the population of isolates from Londrina (cluster A) of

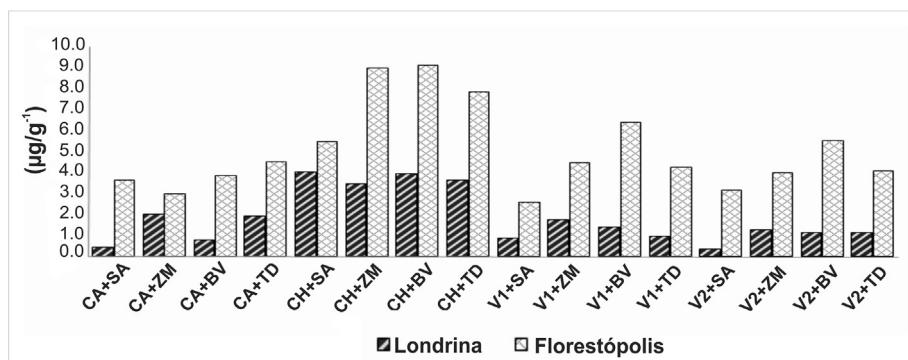
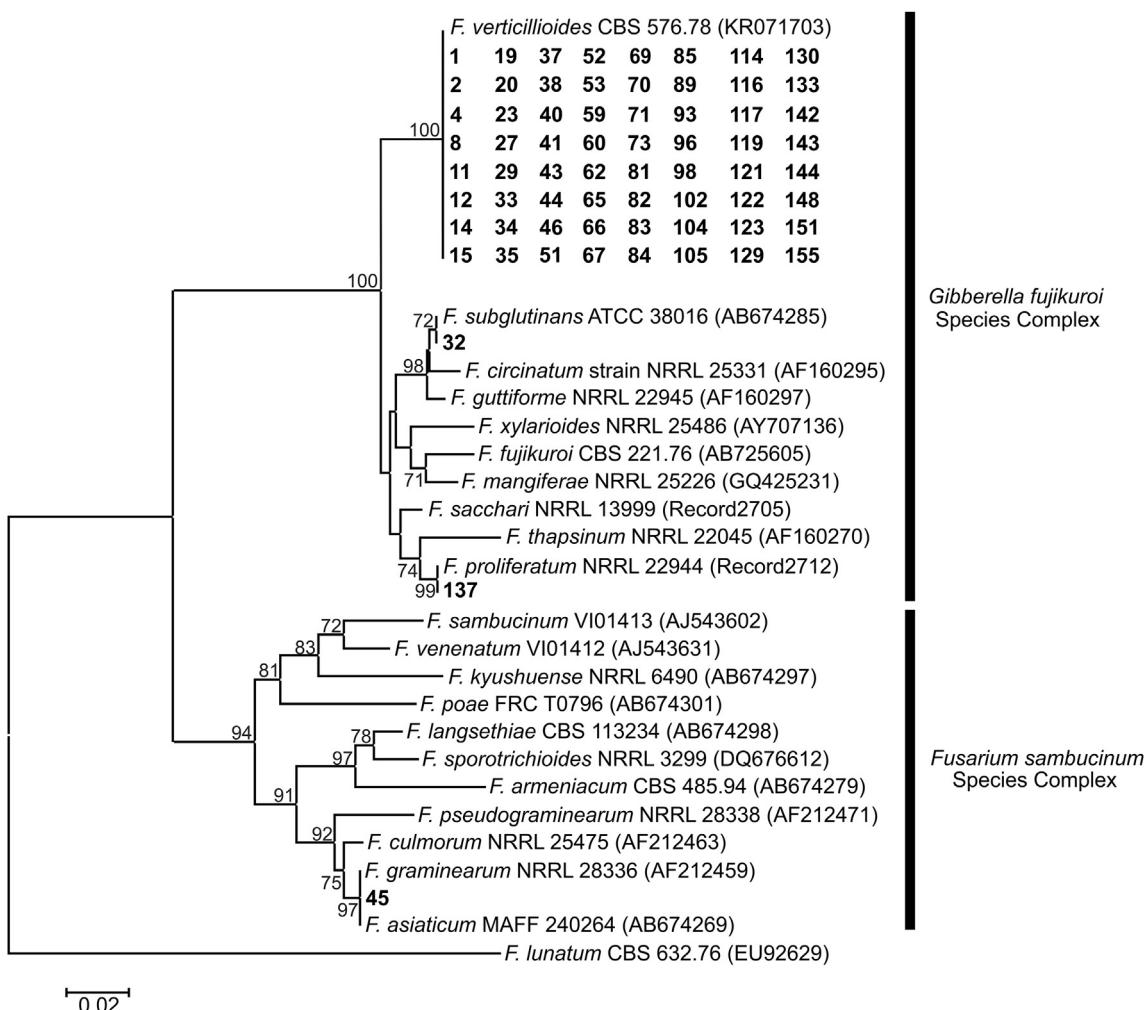


Fig. 1. Total contamination by fumonisins (FB₁+FB₂) in 16 treatments at Londrina and Florestópolis sites. CA (Caiano), CH (Commercial hybrid), V1 (ST509), V2 (ST1309), SA (single application of nitrogen at planting), ZM (nitrogen application at planting combined with a inoculant containing a strain of *Methylobacterium komagatae*), BV (nitrogen application at planting combined with a inoculant containing a strain of *Azospirillum brasiliense*), and TD (complete fertilization, involving nitrogen application at planting and top dressing).

Table 1Incidence of *Fusarium* spp. and level of fumonisins ($FB_1 + FB_2$) in maize kernels.

Variables	Mean values for infection by <i>Fusarium</i> spp. (%)	Mean values of fumonisins ($\mu\text{g g}^{-1}$)			Variation Min. Max.
		FB_1	FB_2	Total ($FB_1 + FB_2$)	
		*	*	*	
Genotype	*				
CA	75.31 a	1.25 a	1.25 a	2.51 a	0.4–4.5
CH	84.37 b	2.82 b	2.99 b	5.81 b	3.5–9.1
V1	75.52 a	1.48 a	1.34 a	2.82 a	0.9–6.4
V2	71.04 a	1.38 a	1.19 a	2.58 a	0.4–5.5
Fertilization practices	NS	NS	NS	NS	
SA	79.47	1.77	1.77	3.54	1.2–7.9
TD	74.68	1.43	1.14	2.56	0.4–5.5
BV	78	2.02	2	4.02	0.8–9.1
ZM	74	1.72	1.89	3.61	1.3–9.0
Location	NS	*	*	*	
Florestópolis	75.67	2.57 a	2.47 a	5.04 a	2.6–9.1
Londrina	77.44	0.89 b	0.92 b	1.81 b	0.4–4.0

NS: not significant, *: significant in the F-test ($p < 0.01$). Means followed by the same letter did not differ significantly in the Tukey test ($p < 0.05$).**Fig. 2.** Phylogenetic tree inferred by the neighbor-joining (NJ) algorithm based on *TEF-1α* gene sequence data. Nodes supported by bootstrap values > 70% are indicated by numeric values. The isolates analyzed in the present study are shown in bold. *Fusarium lunatum* is the outgroup.

that from Florestópolis (cluster B) as shown in Fig. 3. Interestingly, the set of isolates from variety V2 (grown in Florestópolis) share great part of their alleles with those of cluster A. As *F. verticillioides* can be systematically transmitted by seeds (Wilke et al., 2007), we

may speculate that V2 seeds planted in both Florestópolis and Londrina could be previously infected with *F. verticillioides*.

Secondly, a principal coordinate analysis (PCoA) (Fig. S3) has also displayed two main groups, corroborating the attribution test

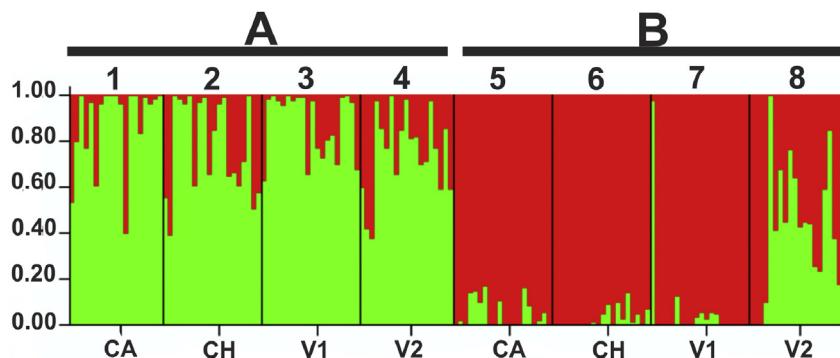


Fig. 3. Structure Bar plot. Groups 1 to 4 (Cluster A) represent isolates from Londrina and groups 5 to 8 (Cluster B) isolates from Florestópolis. CA (Caiano), CH (Commercial hybrid), V1 (ST509), V2 (ST1309). Each color represents a genotypic cluster attributed probabilistically by Structure 2.3.4.

Table 2

Analysis of molecular variance (AMOVA) within and between *F. verticillioides* populations according to geographic origin.

Source of variation	Degree of freedom	Sum of squares	Estimate Variance	Percentage variation	F_{ST}
Between locations	1	447.653	5.45732	18	0.17995*
Within locations	153	3805.031	24.86948	82	
Total	154	4252.684	30.3268		

*p < 0.05.

Table 3

Analysis of molecular variance (AMOVA) within and among *Fusarium verticillioides* populations according to maize genotype.

Locations	Source of variation	Degree of freedom	Sum of squares	Estimate Variance	Percentage variation	F_{ST}
Florestópolis	Among genotypes	3	179.028	2.12547	10.72	0.10718*
	Within genotypes	78	1327.858	17.70477	89.28	
	Total	81	1506.886	19.83024		
Londrina	Among genotypes	3	168.335	1.39701	4.51	0.04510*
	Within genotypes	74	2129.81	29.58069	95.49	
	Total	77	2298.145	30.9777		

*p < 0.05.

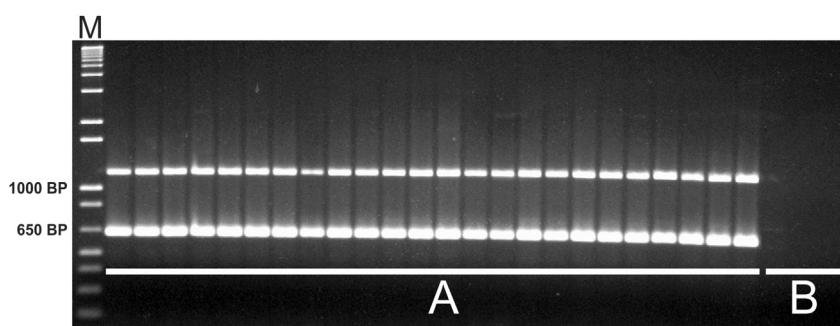


Fig. 4. Multiplex PCR results obtained with specific primers for *FUM1* (635 bp) and *FUM8* (1152 bp) genes. *F. verticillioides* isolates positive (A) and negative (B) for gene amplifications. (M) DNA Ladder 1 kb.

results and showing a clear grouping according to maize growing location.

Finally, the results of the AMOVA, performed for separating the fungal isolates according to growing location, revealed significant genetic differentiation ($F_{ST} = 0.18$), indicating that the allele frequencies are different in the two populations (Table 2). In dissonance with our results, some previous studies on *F. verticillioides* populations, also isolated from maize, revealed no significant association between genetic variation and geographic origin (Covarelli et al., 2012; Reynoso et al., 2009; Tshehay et al., 2016).

Then, the results of the AMOVA performed for separating the

isolates according to plant genotype also revealed significant genetic differentiation in both sites (Table 3).

3.4. Toxigenic potential of *Fusarium* spp.

The synthesis of a carbon chain and subsequent amination are two key stages in fumonisin biosynthesis. The first stage is catalyzed by a polyketide synthase (PKS) coded by the *FUM1* gene and the polyketide amination is catalyzed by an α -oxyamine synthase coded by the *FUM8* gene (Proctor et al., 1999, 2003, 2013; Seo et al., 2001). The presence of both genes is a good indication of the

toxigenic potential of the fungal isolate.

Amplification products obtained by PCR (Fig. 4) revealed that most of *F. verticillioides* isolates (95%) did contain the *FUM1* (680 bp) and *FUM8* (1152 bp) genes, suggesting the toxigenic potential of the Brazilian isolates herein evaluated.

4. Conclusions

In conclusion, the two maize varieties and the Caiano landrace exhibited much lower levels of fungal infected kernels and fumonisin contamination than those of the commercial hybrid cultivated in two Southern Brazilian regions. The molecular analysis revealed that *Fusarium verticillioides* was the prevalent species in those maize kernels, and fungal isolates were genetically diverse. Interestingly, the population was genetically structured, and differentiation was primarily associated with geographic origin, but maize genotypes also contributed to substructuring the populations. Overall, our study has generated valuable information for understanding the diversity and toxigenic potential of *Fusarium* spp. associated with cultivated maize in Brazil.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.cropro.2017.05.020>.

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