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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 B_1 and B_2 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 B_1 and B_2 were detected in all samples, with levels ranging from 0.4 to 9.1 $\mu g \times g^{-1}$. 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.

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-Montaño 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 (FB_1) and B2 (FB_2) 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 FB_1 and FB_2 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 toxigenic 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; Tsehaye 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, totalizing 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 2.5×10^9 cells mL^{-1}, the liquid inoculant was prepared (composition patent pending). The final cell concentration in the inoculant was 10^9 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 B_1 and B_2

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 N_2 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-phthaldialdehyde (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 (FB_1 and FB_2) 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 5μ 100 Å column (4.6 × 250 mm) (Phenomenex, USA). Excitation and emission wavelengths were 335 and 450 nm, respectively. The eluent was CH_3OH: 0.1M NaH_2PO_4 (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