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ORIGINAL ARTICLE

Molecular and mycotoxin characterization of *Fusarium graminearum* isolates obtained from wheat at a single field in Argentina

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Abstract *Fusarium graminearum* is the primary causal agent of Fusarium head blight of wheat in Argentina. This disease affects yield losses and quality of grains, reducing the wheat end-use, also causing mycotoxin contamination. In this study, the genetic variability and deoxynivalenol (DON) potential/production of *F. graminearum sensu stricto* (*s.s.*) isolates obtained from wheat samples of the 2009, 2010, and 2011 growing seasons from a single location in Argentina were evaluated. The genetic variability detected using inter-simple sequence repeat (ISSR) was analyzed in relation to the in vitro deoxynivalenol production, the main monitored and quantified mycotoxin according to the current regulations for the international marketing of cereals. Of the 68 *F. graminearum s.s.* isolates obtained in this study, 95 % showed a different banding pattern with ISSR molecular markers and a high variability was detected within the population. However, no clustering was found in relation with year or DON production. All isolates amplify for the DON-related gene and a high variability in DON production was observed among the isolates, with production values between non-producers and 1741 μg/g. The results suggest that the *F. graminearum s.s.* population varies significantly in both genetic structure and toxin production in a limited sampled area.

Keywords *Fusarium graminearum* · Wheat · Genetic variability · Deoxynivalenol

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Introduction

Latin American countries in general and mainly Brazil and Argentina are important wheat producers in the world, with Argentina being among one of the major wheat exporters (Calzada 2014; Reynoso et al. 2011). Fusarium head blight (FHB) is one of the most severe fungal diseases affecting wheat crops worldwide, including those in Argentina (Galich 1996; Lori et al. 2003). Many members of the *Fusarium graminearum* species complex (FGSC) are associated to this disease, with *Fusarium graminearum sensu stricto* (*s.s.*) being the main causal pathogen reported in Argentina (Alvarez et al. 2011; Castañares et al. 2014; Ramirez et al. 2007; Sampietro et al. 2010). In this complex, 16 different phylogenetically species have been recognized (Aoki et al. 2012; Sarver et al. 2011). FHB directly affects yield losses and quality of grains, reducing the wheat end-use, also causing mycotoxin contamination (Matny 2015). At least 20 FHB epidemics of wheat have been registered in the last 50 years in Argentina, with variable intensity (Kikot et al. 2011; Malbrán et al. 2012; Moschini and Fortugno 1996).

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Even though different strategies have aimed to control the effects of the disease, such as chemical control (Lechoczki-Krsjak et al. 2008), biological control (Mourelos et al. 2014; Zhao et al. 2014). and the development of resistant cultivars (Cainong et al. 2015). satisfactory levels of control have not yet been attained. It is noteworthy that knowledge of the genetic variability of this pathogen is relevant to understand its epidemiology and evolutionary potential, which provides useful information to control and reduce the devastating effects of the disease (Zeller et al. 2003).

Several molecular techniques have been used to analyze *F. graminearum* isolates and to provide information about the population structure worldwide, such as amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), variable number tandem repeat (VNTR), and inter-simple sequence repeat (ISSR) (Karugia et al. 2009; Leslie et al. 2007; Llorens et al. 2006). The population structure of *Fusarium* species in South America is poorly understood compared to other production regions. The studies recorded in Argentina regarding genetic variability among *F. graminearum s.s.* isolates have been based mainly on AFLP molecular markers (Alvarez et al. 2011; Ramirez et al. 2007). The analysis based on ISSR-polymerase chain reaction (PCR) molecular markers is considered a reliable methodology in population genetic studies of fungi, in which the selection of ISSR primers does not require previous knowledge of the sequence and generates specific and reproducible patterns due to the highly stringent conditions of the reaction (Mishra et al. 2003).

The mycotoxins most associated with members of the FGSC are deoxynivalenol (DON) and nivalenol (NIV), which have several adverse effects on animal and human health, such as gastrointestinal disorders and severe depression of the immune system, promoting the development of secondary infections (Pestka 2007; Pestka and Smolinski 2005). In South America, the main mycotoxin is DON, belonging to type B trichothecenes, whose presence is monitored and quantified due to current regulations for the international marketing of cereals (Astolfi et al. 2012; Castañares et al. 2014; Umpiérrez-Failache et al. 2013). In our country, the DON genotype/chemotype seems to be predominant in the wheat-cropping area (Alvarez et al. 2009; Malbrán et al. 2014; Ramirez et al. 2007).

Different studies on genetic variability and/or mycotoxin capacity at the genome level/in vitro production of *F. graminearum s.s.* have been carried out (Alvarez et al. 2009; Astolfi et al. 2012; Ramirez et al. 2007). However, the majority of them were conducted to reflect the genetic or mycotoxin variability among different wheat cultivated areas in a region or in a country, and little is known about the variability in a small and single wheat production area (Karugia et al. 2009). The purpose of this work was to analyze the genetic variability and both DON potential (genetic level) and in vitro production among *F. graminearum s.s.* isolates obtained from a single field.

Materials and methods

Biological materials

Thirty-three wheat samples (50 g each) obtained during the 2009, 2010, and 2011 wheat growing seasons were harvested and threshed manually. The samples belong to the national wheat breeding program, in an experimental field of 1 ha of Marcos Juárez-INTA (32°42′S; 62°06′W), province of Córdoba, Argentina, which is one of the most important producer and often studied areas of wheat cultivars in the country. Conventional tillage operations according to standard production practices on fields, in an Argiudol soil, were made.

Isolation of *F. graminearum*

For *F. graminearum* isolation, 60 grains per wheat sample were surface-sterilized with 10 % *v*/*v* sodium hypochlorite solution and plated onto Petri dishes containing wet filter paper in darkness at 25 °C for 7 days. Grains that showed fungal presence were transferred to potato dextrose agar (PDA) medium. Mycelium was subcultured on Spezieller Nährstoffarmer agar (SNA) medium for 15 days and then conidium suspension was transferred to water agar (WA) medium for 16 h. A single conidia was picked and transferred to PDA. Identification was carried out by cultural characterization on PDA and carnation leaf agar (CLA) by means of the keys of Gerlach and Nirenberg (1982). Burgess et al. (1994). and Leslie and Summerell (2006).

Molecular characterization

Molecular identification and potential production of DON

Total genomic DNA from presumable *F. graminearum* isolates was extracted using the cetyltrimethylammonium bromide (CTAB) method (Stenglein and Balatti 2006) and DNA concentrations were calculated using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). To confirm morphological identifications, an *F. graminearum*-specific PCR assay was performed for the isolates using primers Fg16F and Fg16R, according to Nicholson et al. (1998). These primers are not completely specific to *F. graminearum s.s.*, but they give products of different size. *Fusarium graminearum s.s.* (Castañares et al. 2014, used as control) gives a product of about 400 bp, while *F. asiaticum* (NRRL 13818, used as control) gives a PCR product of about 550 bp, *F. meridionale* (NRRL 28436, used

as control) gives a product of about 500 bp, and no amplification was expected for *F. boothii* (NRRL 26916, used as control), according to Waalwijk et al. (2003). Mugrabi de Kuppler et al. (2011). Castañares et al. (2014, 2015). and Covarelli et al. (2015). The species used as a control were selected according to their presence in South America (van der Lee et al. 2015). DON genotype determination was conducted by amplifying a portion of the *Tri*13 gene (Chandler et al. 2003). *Fusarium poae* and *F. pseudograminearum* DNA were used as negative controls for the specific PCR reactions.

ISSR markers

The ISSR-PCR was performed using five ISSR primers: Biolab H [$(GCC)_5$], Biolab D [$(AG)_8$], Biolab F [$CT(GA)_8$], Biolab J [$(CAC)_5$], and Biolab E [$CTC(GT)_8$]. PCRs were performed in an XP Thermal Cycler (Hangzhou Bioer Technology Co., Ltd., Hangzhou, China), according to Dinolfo et al. (2010). Cluster analysis based on the Dice coefficient was realized on the similarity matrix employing the unweighted pair group method with arithmetic mean (UPGMA) algorithm (Sneath and Sokal 1973). NTSYSpc version 2.1 was used for the analysis (Rohlf 1998). An analysis of molecular variance (AMOVA) was realized using Arlequin 2000 software (Schneider et al. 2000). DNA from a *F. pseudograminearum* isolate was used as the outgroup (Castañares et al. 2013).

DON production

Sample preparation

DON analyses were carried out following the methodology proposed by Cooney et al. (2001). with some modifications. Wheat grains were irradiated with 10–12 kGrays of gamma irradiation. Water activity (a_W) was measured using an Aqualab Series 3 (Labcell Ltd., Basingstoke, Hants, UK) and then rehydrated to get a value of 0.995. The wheat grains were placed on Petri dishes forming a monolayer (20 g) and then inoculated with a 4-mm-diameter agar disk taken from the margin of a 7-day-old growing colony of each isolate on PDA and incubated at 28 °C. Cultures were carried out in triplicate. After 28 days of incubation, the samples were dried in a forced air oven and finely ground with a propeller grinder Arcano (Instrumental Pasteur, Buenos Aires, Argentina). Fifteen grams of each sample were mixed with 40 mL of acetonitrile:methanol (14:1) and shaken at high speed for 1 h. Then, an aliquot of 2 mL of each isolate was taken and added to a cleanup cartridge, containing a layer of glass wool and 500 mg of a mixture of alumina:carbon (20:1). DON was eluted from the cartridge with 500 μL of acetonitrile:methanol:water (80:5:15), evaporated to dryness under nitrogen gas and resuspended in methanol:water (95:5). Cultures with uninoculated grains were employed as controls.

HPLC conditions

DON detection and quantification was performed using high-performance liquid chromatography (HPLC, Waters 717 plus Autosampler) with a UV detector (220 nm) (Palazzini et al. 2007). The chromatographic separations were carried out on a C18 reverse phase column (250×4.6 mm, 5-μm particle sizes, Waters). The mobile phase used a mixture of water:methanol (88:12) with a flow rate of 1.5 mL min^{-1}. Quantification was performed by measuring the peaks (Empower software; Waters Corporation, Milford, MA, USA) and extrapolation to a calibration curve was obtained using DON standard solutions of 1–5 μg mL^{-1} in methanol:water (5:95) (Sigma Aldrich Co., St. Louis, MO, USA; purity >99 %). The purity of the peak was determined by performing a spectral homogeneity test with the same software. This test showed values of limit of detection (LOD)=0.15 μg/g, limit of quantification (LOQ)=0.50 μg/g, and recovery percentage of 96 %. In order to analyze the results, the isolates were divided into four DON production levels: not detectable (ND), low producers (lower than 50 μg/g), medium producers (50–150 μg/g), and high producers (higher than 150 μg/g).

Results and discussion

Sixty-eight *Fusarium graminearum s.s.* isolates were obtained from 24 wheat samples, with amplified fragments of about 400 bp (Fig. 1). No *Fusarium graminearum s.s.* were obtained from the remaining nine wheat samples. Most *F. graminearum s.s.* isolates were obtained from the 2010 wheat growing season (66). Only one isolate from the 2009 and another from the 2011 wheat growing seasons were identified as *F. graminearum s.s.* Consequently, it became actually a point analysis on the *Fusarium* population at one site (Marcos Juárez) in 2010.

Higher percentages of the presence of *F. graminearum s.s.* were reported by other authors in Argentina. Ramirez et al. (2007) and Alvarez et al. (2011) found that 100 and 97 % of the isolates obtained from locations in the major wheat production area of Argentina were members of this species, respectively. Similar results were obtained in Uruguay by Pan et al. (2013). who identified all isolates obtained as *F. graminearum s.s.* Moreover, values closer to those observed in our research were reported for southern Brazil by Astolfi et al. (2012). which were near to 74 %.

The occurrence of FGSC members over time is strongly related to competition among them, according to the severity degree of FHB (Xu et al. 2007). Although different members of the FGSC can be found in much of the geographical area

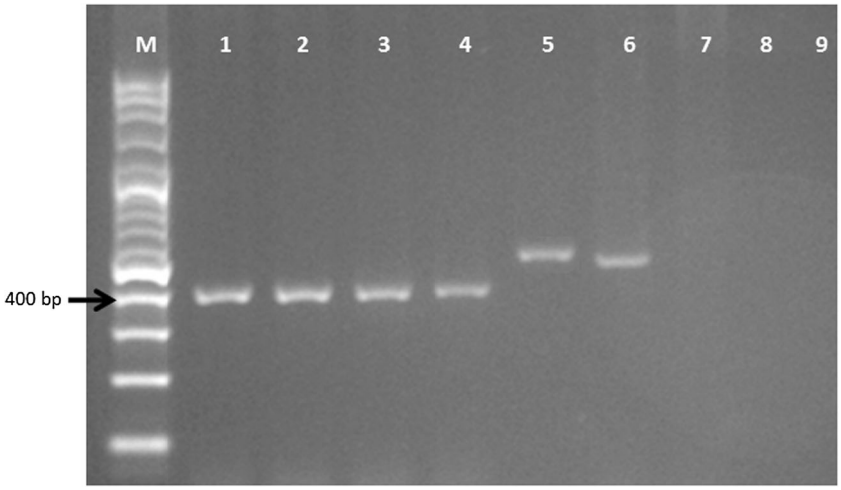

Fig. 1 Amplification patterns. M: molecular marker; 1–4: *F. graminearum s.s.*; 5: *F. asiaticum*; 6: *F. meridionale*; 7: *F. boothii*; 8: *F. pseudograminearum*; 9: *F. poae*

affected by the disease, *F. graminearum s.s.* is the most dominant around the world (O'Donnell et al. 2000, 2004, 2008; van der Lee et al. 2015). Furthermore, the predominance of one species over another could be attributed to environmental conditions and/or host preference (Del Ponte et al. 2015; Osborne and Stein 2007; Xu et al. 2007, 2008).

At the moment, among FGSC species, only *F. graminearum s.s.* has been isolated from wheat grains in Argentina (Alvarez et al. 2011; Ramirez et al. 2007). However, different species of the complex were isolated from wheat in Uruguay and Brazil, where subtropical environmental conditions favor FGSC diversity (Astolfi et al. 2012; Umpiérrez-Failache et al. 2013).

The ISSR dendrogram defined 65 haplotypes (Fig. 2). The isolates were resolved into two clusters (I, II), with an average similarity among groups of 51 %. The *F. pseudograminearum* strain used as the control appeared alone in a third cluster (III). The isolates distribution in the dendrogram was dispersed, showing no clustering according to the origin of wheat or with DON production. The genetic variability determined by AMOVA showed that 99 % of the isolates are different. The use of molecular markers has also been demonstrated to be a useful tool in the analysis of genetic diversity in populations of *F. graminearum* in South America. In Argentina, Ramirez et al. (2007) and Alvarez et al. (2011) detected around of 98 and 100 % of genetic variability, respectively. In addition, Astolfi et al. (2012) showed 93 % of variability in south Brazil, although they used *F. graminearum s.s.* isolates from different sampled areas of wheat.

In general, variability studies for FGSC members have been conducted from several geographical locations (Alvarez et al. 2009, 2011; Boutigny et al. 2011, 2014; Qiu et al. 2014; Sampietro et al. 2010). By contrast, there are a scarce number of authors that have analyzed the genetic variability of the species among a limited number of localities (Astolfi et al. 2012; Guo et al. 2008; Mishra et al. 2004; Ramirez et al. 2006b, 2007) or even at a single field location (Karugia et al. 2009). In such a case, as well as being observed in the present study, a high genetic variability can be found in a single geographical area. This variability could be attributed to several factors. Ramirez et al. (2007) suggested that a high level of genotypic diversity with relatively few clones is a consequence of frequent outcrosses, enough to maintain a great deal of genetic heterogeneity in the population, able to rapidly synthesize a multilocus response to change in selection pressures. In addition, the co-existence of different members of the FGSC provides a potential for hybridization and the formation of new genotypes that could affect pathogenicity, host range, or toxin production (Alvarez et al. 2011; Karugia et al. 2009). Furthermore, spores are easily transported among different locations, increasing diversity (Schmale et al. 2006).

Genotype analysis showed that all *F. graminearum s.s.* amplified the fragment of 282 bp corresponding to the *Tri*13 gene involved in the DON trichothecene biosynthesis (Chandler et al. 2003). The determination of DON production showed significant differences among isolates ($p<0.05$), observing values that ranged from not detectable (ND) to 1741 μg/g, demonstrating a high intraspecific variability in their toxin production profile, even for isolates coming from a single location. Four isolates (5.9 %) belonged to the ND group, 51.5 % from the low producers, 19.1 % from the medium producers, and 23.5 % from the high producers (Fig. 3). However, the predominant group belonged to values less than 50 μg/g, with these values being the most frequently reported (Alvarez et al. 2009; Palazzini et al. 2007; Ramirez et al. 2006a).

There are few studies that use wheat as a substrate to analyze DON production in vitro by *F. graminearum* (Hope et al.

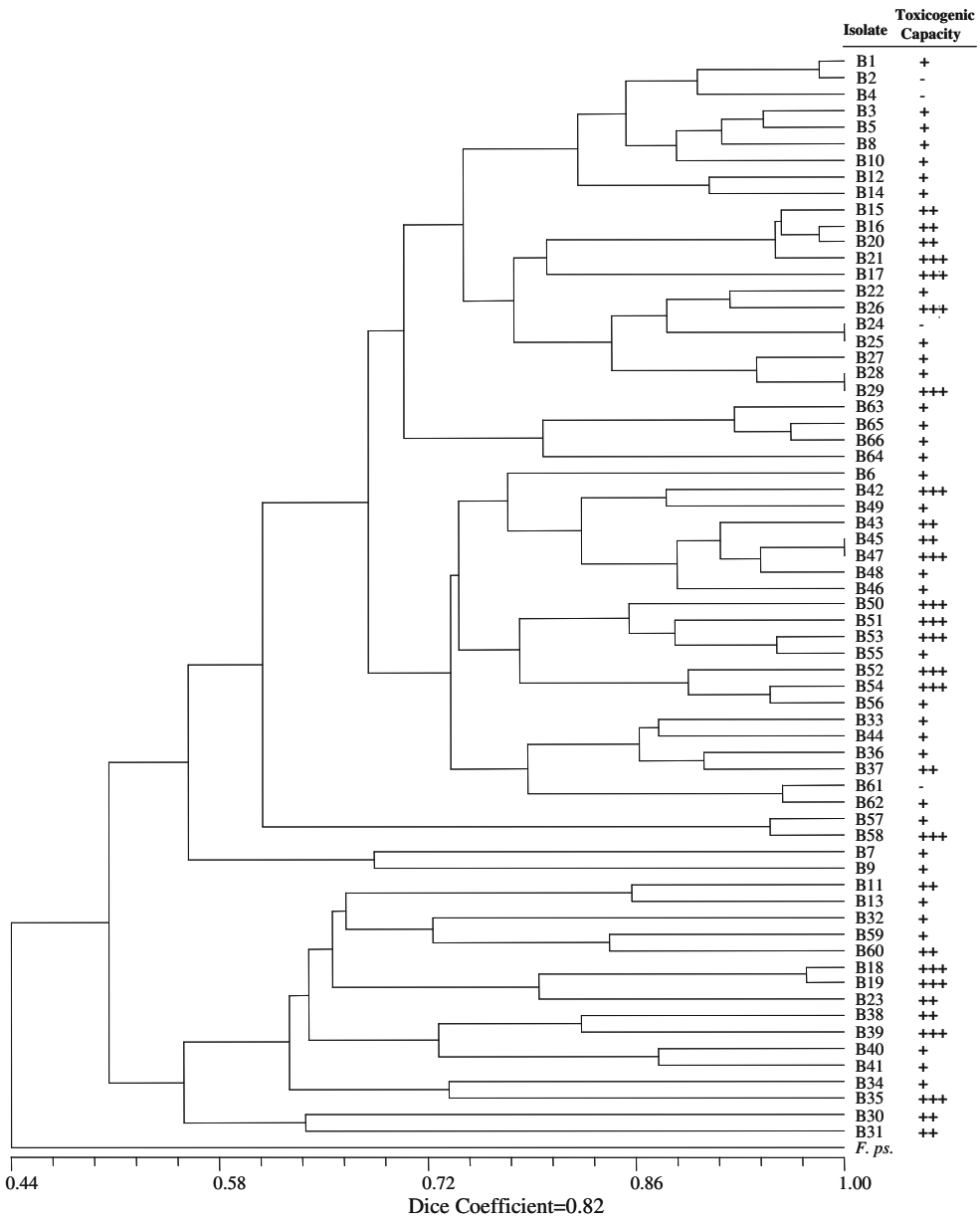

Fig. 2 Dendrogram obtained by ISSR markers showing cluster grouping of the 66 *F. graminearum s.s.* isolates from the year 2010 plus *F. pseudograminearum* strain (*F. ps*) and the DON production. (−): Not detectable; (+): low producers; (++): medium producers; (+++): high producers

2005; Ramirez et al. 2006a, b). All these reports showed values of production <10 μg/g. At the genetic level, all isolates were potentially DON producers, similar to those reported previously by Burlakoti et al. (2011) and Castañares et al. (2014). Differences between molecular assays and in vitro production could be explained as PCR shows the capacity, at the genome level, to produce a specific toxin, whereas the production is dependent on the substrate and environmental conditions of the assay (Castañares et al. 2014; Somma et al. 2014).

Moreover, when Goswami and Kistler (2005) evaluated the variation in mycotoxin production and aggressiveness among isolates belonging to different species from the FGSC, they observed that the high level of variability for both characters was specific to each of the isolates tested and independent of the species considered.

In conclusion, our results showed that, even at a single and small area, the population of *F. graminearum s.s.* presents a high variability in their genomic pool and DON production. This observed level of diversity could be considered as one of the causes behind the periodical emergence of this pathogen in different areas around the world. Furthermore, the high variability observed in the toxigenic production could be considered as another indicator of this genetic diversity. This study reflects that continuous monitoring not only at the regional level, but also at the single field level, is needed in order to understand the *F. graminearum s.s.* population.

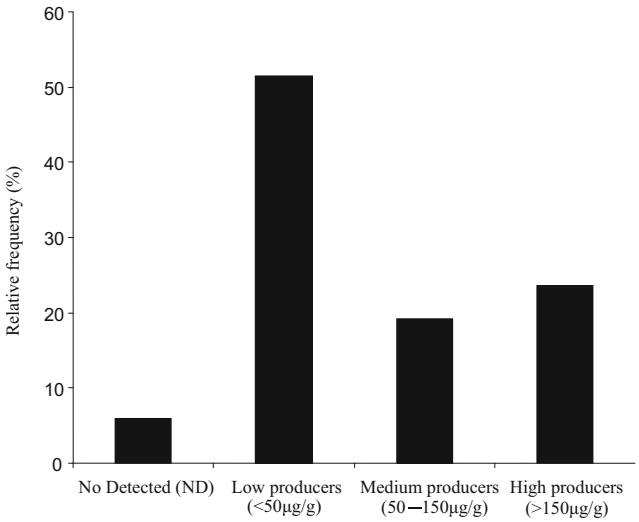

Fig. 3 Relative frequency of isolates distributed in each DON production range

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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