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Trichothecene genotype and genetic variability of *Fusarium graminearum* and *F. cerealis* isolated from durum wheat in Argentina

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Abstract *Fusarium* head blight (FHB) is one of the most important fungal diseases affecting wheat worldwide and it is caused mainly by species within the *Fusarium graminearum* species complex (FGSC). This study evaluated the presence of FGSC in durum wheat from the main growing area in Argentina and analyzed the trichothecene genotype and chemotype of the strains isolated. Also, the genetic variability of the strains was assayed using ISSR markers. Molecular analysis revealed that among the strains isolated and identified morphologically as *F. graminearum*, there were 14 strains identified as *F. cerealis*. Also, it revealed that durum wheat grains were mostly contaminated by *F. graminearum*, being this the only species reported so far, within the FGSC, affecting durum wheat in Argentina. Analysis of molecular variance (AMOVA) indicated a high genetic variability within rather than between *F. graminearum* populations. All *F. graminearum* strains presented 15ADON genotype and were able to produce DON while all *F. cerealis* strains presented the NIV genotype and most of them were able to produce this toxin. The finding of *F. cerealis* in durum wheat grains indicates the need for investigating if this fungus is the responsible for the NIV contamination found in wheat in Argentina.

Introduction

Fusarium Head Blight is one of the most destructive and economically important fungal diseases of wheat and other small grain cereals worldwide. Under favorable conditions (high humidity and warm temperatures), yield losses can reach up to 50% (Parry et al. 1995). The disease can severely reduce grain yield and quality and the infected grains could be contaminated with mycotoxins such as trichothecenes. This results in difficulties for wheat trading, besides the threat to human and animal health. The disease is caused mainly by *Fusarium culmorum* and species within the *Fusarium graminearum* species complex (FGSC) which is comprised of 15 formally described phylogenetic species and one additional species which was informally recognized based on genealogical exclusivity and conidial morphology on SNA (Aoki et al. 2012). Within these species, *F. graminearum* and *F. asiaticum* are the main

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species associated with the disease worldwide. *Fusarium graminearum* is the main pathogen isolated from scabby grains in North America, Europe and South America, and *F. asiaticum* has been found to be the most important FHB pathogen in China, Japan and Korea (Aoki et al. 2012; Astolfi et al. 2012; Ramirez et al. 2007). Also, other *Fusarium* species such as *Fusarium avenaceum*, *Fusarium verticilloides*, *Fusarium cerealis*, *Fusarium oxysporum* and *Fusarium poae* could contribute to the disease (Bottalico and Perrone 2002).

Mycotoxins are secondary metabolites produced by filamentous fungi and trichothecenes are the most common mycotoxins found in cereals (Desjardins 2006). Species within the FGSC are capable of producing, usually, one of the three trichothecene profiles: (i) deoxynivalenol and 3-acetyldeoxynivalenol (3ADON chemotype), (ii) deoxynivalenol and 15-acetyldeoxynivalenol (15ADON chemotype), or (iii) nivalenol and its acetylated derivatives (NIV chemotype) (Ward et al. 2002). The 15ADON chemotype dominates in North America, South America, western, southern and central Europe, southern Russia and South Africa (Astolfi et al. 2012; Boutigny et al. 2011; Pan et al. 2013; Pasquali et al. 2016; Puri and Zhong 2010; Reynoso et al. 2011), while 3ADON chemotype dominates in northern Europe, north-western Russia, China and Japan (Pasquali et al. 2016; Wang et al. 2011; Yli-Mattila and Gagkaeva 2010; Zhang et al. 2012). PCR assays based on the amplification of gene portions coding for key enzymes involved in trichothecene biosynthesis have been developed in order to rapidly determine the trichothecene genotypes of the FGSC strains (Quarta et al. 2006; Ward et al. 2002).

Durum wheat (*Triticum turgidum* L. var. *durum*) is an important small grain cereal, used for human consumption. In Argentina this cereal is mainly used for pasta production. In the last few years the national production of pasta has increased to 40% and in 2013 it reached 352,062 tons. The consumption per capita was estimated in 8.27 kg/year (UIFRA 2014). At present, durum wheat production covers about 64,000 ha, which represent only 1% of the total wheat produced in our country (MAGyP 2015). The main growing area is located at the south of Buenos Aires province which belongs to the Pampas region. It is subdivided into three agroecological areas with different climatic conditions: southeast (humid), mid-south (sub-humid) and southwest (semi-arid).

Wheat is an important crop in the human diet, therefore its quality and safety is of major concern. So far, crop management and chemical control are partly effective to control the disease. Hence, the use of resistant cultivars plays a key role in integrated *Fusarium* control and the prevention of mycotoxin contamination. However, the durum wheat cultivars used at present in Argentina show low levels of FHB resistance. Several epidemics have occurred in the main growing area in 1963, 1976, 1978 and 1985, with crop losses as high as 70%. Also during 2001 early severe symptoms were observed (Carranza et al. 2008).

Several studies on *F. graminearum* populations have been carried out worldwide, including South America (Ramirez et al. 2007; Astolfi et al. 2012; Pan et al. 2016). In Argentina, there are several studies on *F. graminearum* populations; however, they all focus on strains isolated from common wheat and from the main growing area of this cereal. Little is known about *Fusarium* species causing FHB in durum wheat and from the region where this crop is cultivated in the south of Buenos Aires province which has a cooler climate.

The objectives of this study were: (a) to evaluate the presence of *F. graminearum* species complex in durum wheat grains from the main growing area of Argentina, b) to determine the trichothecene genotype of the strains and their capability to produce DON in vitro, and c) to analyze the strain genetic variability with ISSR markers.

Materials and methods

Sampling, strain isolation and morphological identification

One hundred twenty-six durum wheat grain samples were obtained from commercial cultivars (developed by Buck Company, CEI Barrow and ACA seeds) and experimental lines from the 2008, 2009, 2010 and 2012 harvest years in different localities (Balcarce, Barrow, Bordenave, Cabildo, La Dulce, Miramar and Pieres) from the main durum wheat producing area in Argentina, south of Buenos Aires province (Fig. 1, Table 1). Localities belong to different areas, southeast area (Balcarce, Miramar and Pieres), mid-south area (La Dulce and Barrow) and southwest area (Cabildo and Bordenave). These areas have different climatic conditions. The average annual rainfall is 900 mm for southeast area, 800 mm for mid-south area and 600 mm for

Fig. 1 Buenos Aires province map indicating the localities and areas where durum wheat samples were obtained
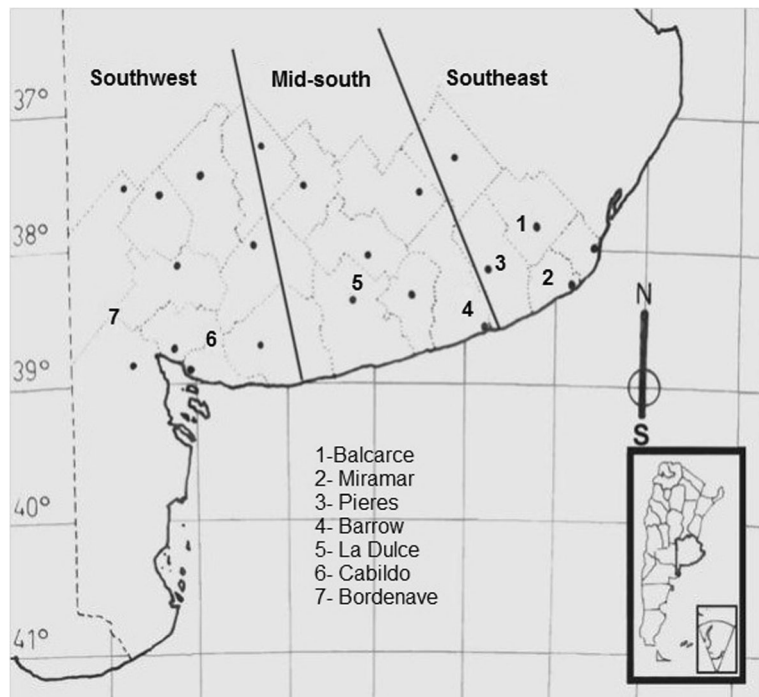
southwest area. Sowing dates were between June and July and harvest between December and January of each year. The soil types of the fields were Typic or Petrocalcic Argiudols and the fungicides utilized, if necessary, were Azoxistrobina + Cyproconazole. The plant material was collected along transects, with sampling stations within fields being 5 m from each other. At each sampling station, five or six wheat heads were selected. From these samples, a subsample of 500 g was taken and immediately stored at 4 °C.

From each subsample 100 wheat grains were plated (10 grains per Petri dish) onto a modified pentachloronitrobenzene medium (PNBC). The PNBC plates were incubated at 24 °C for 7 days under 12/12 h photoperiod cold white and black fluorescent lamps. *Fusarium* species developing from the grains were then identified according to previous guidelines (Leslie and Summerell 2006). Representative cultures of the species isolated were grown from single conidia for 10–14 days on Petri dishes of carnation leaf agar (CLA) and potato dextrose agar (PDA) slants, at 24 °C with a 12/12 h photoperiod under cold white and black fluorescent lamps. Strains identified morphologically as *F. graminearum* were used for further analysis.

Table 1 Locality, region, sampling year and strains isolated from durum wheat samples from the main Argentinean growing area

Locality	Region	Sampling year	*F. graminearum* strains isolated	*F. cerealis* strains isolated
1-Balcarce	Southeast	2009, 2012	6	0
2-Miramar	Southeast	2009, 2010, 2012	9	3
3-Pieres	Southeast	2009, 2010	9	6
4-Barrow	Mid-south	2009, 2010, 2012	3	2
5-La Dulce	Mid-south	2008, 2009, 2010, 2012	21	3
6-Cabildo	Southwest	2010, 2012	0	0
7-Bordenave	Southwest	2010	0	0

DNA extraction

Total genomic DNA was extracted using the cetyltrimethylammnonium bromide (CTAB) method as described by Leslie and Summerell (2006). In brief, all *F. graminearum* strains were grown in Wickerham medium, containing 40 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract and water up to 1000 ml and incubated on an orbital shaker (150 rpm) for at least three days at 25 ± 1 °C. The resulting mycelia were harvested by filtration through non-gauze milk filters (Ken AG, Ashland, Ohio). Excess water was removed by blotting mycelia between clean paper towels, and dried mycelia were stored frozen at −20 °C until ground. Genomic DNA quality was determined by electrophoresis and quantified using a fluorometer (Qubit™-Invitrogen, Buenos Aires, Argentina).

ISSR analysis

From a collection of 15 primers, four, ISSR6, ISSR7, ISSR8 and ISSR10, were used to assess diversity of 62 putative *F. graminearum* strains (Table 2). These primers were selected based on the number and intensity of bands and on their ability to consistently amplify the same fragment(s) from a given strain. All amplifications were performed in a 25 μl final volume containing 15 ng of genomic DNA, 1X reaction buffer, 0.7 μM of each primer, 0.2 mM dNTPs, 2.5 mM of $MgCl_2$, 1.25 U Taq DNA polymerase (Invitrogen, Buenos Aires, Argentina). A negative control, containing all reagents and primers but no fungal DNA, was included with every set of reactions and each reaction was performed at least twice. Amplifications were conducted in a PTC-2000 Thermal Cycler (MJ Research Inc., Watertown, MA). The PCR conditions were: 94 °C, 7 min then 33 cycles of 94 °C for 60s, annealing temperature according to the sequence of the primer used (Table 2) for 75 s, 72 °C for 4 min, followed by a final extension step

of 7 min at 72 °C. All amplicons generated were resolved on 1.5% agarose gels. The gels were stained with ethidium bromide (5 μg/ml) and visualized under UV light. The size of the DNA fragments was estimated by comparing the DNA bands with a 100 bp DNA ladder (Invitrogen, Buenos Aires, Argentina). Gel images were photographed using a gel documentation system (MiniBIS Pro, DNR). The profile of reference strains was used for comparison with the individual strain profiles. Seven reference *Fusarium* strains were included in the analysis: *F. culmorum* 11,452, *F. cerealis* 11,451 and *F. pseudograminearum* 11,436 (Kindly supplied by Dr. John F. Leslie, Kansas State University, USA) and *F. graminearum* NRRL 31084, *F. meridionale* NRRL 11794, *F. cortaderiae* NRRL 29297 and *F. boothi* NRRL 26916 which belong to the FGSC. These last four species were selected considering that they are the only species within the complex reported in Argentina so far (Barros et al. 2012; Ramirez et al. 2007; Sampietro et al. 2011).

ISSR fingerprints were manually scored as presence (1) or absence (0) of a DNA band, and recorded in a binary format. All bands were scored, including those that were monomorphic. Bands migrating at the same position were assumed to be homologous and to represent the same allele and locus. Bands with different mobility were treated as independent loci with two alleles (present or absent). Irresolvable bands and missing data were treated as missing data. To test the reproducibility of the amplification patterns, all DNA amplifications were repeated at least twice to ensure reproducibility of primers. The bands were considered reproducible and scorable only after observing and comparing them in two separate amplifications for each primer. Clear and intense bands were scored while faint bands against background smear were not considered for the further analysis.

Banding pattern analysis

To estimate the genetic distance between individuals, a similarity coefficient (S) was calculated using the formula: $S = 2Nxy/(Nx + Ny)$, where Nx and Ny are the number of fragments amplified in strains x and y, respectively, and Nxy is the number of fragments shared by the two strains (Nei and Li 1979). The genetic distance (D) was derived from similarity coefficients as $D = 1 - S$. A genetic distance matrix was generated from the compiled ISSR data. A dendrogram was

Table 2 ISSR primers used in this study

Primer name	Sequence	Annealing temperature
ISSR6	$CTC(GT)_8$	53 °C
ISSR7	$AG(CTC)_5$	51 °C
ISSR8	$CT(GA)_8$	53 °C
ISSR10	$(GA)_8C$	47 °C

constructed by cluster analysis based upon the UPGMA (Unweighted pair-group method using arithmetic averages) algorithm and the cophenetic correlation coefficient (CCC) was chosen to indicate the level of distortion between the similarity matrix and cluster analysis. NTSYSpc version 2.0 (Numerical Taxonomy System, Exeter Software) was used to perform these analyses. The statistical support for different branches was estimated by bootstrapping with 1000 replicates using PAUP* version 4.0 (Swofford 2001). To analyze *F. graminearum* genetic variation, the strains were grouped into two populations based on the area where they were isolated: southeast population and mid-south population, since any strain was isolated from the southeast area. Genetic variation was evaluated by analysis of molecular variance (AMOVA) in GENALEX 6.2 (Peakall and Smouse 2006). ΦST, an analogue of fixation index (Fst) but more suitable for binary and haploid data, was used to estimate genetic differentiation between populations.

Fungal DNA amplification and sequencing

DNA sequencing approach was utilized to confirm the identity of the strains obtained by the ISSR analysis. A partial region of the Elongation Factor 1 alpha gene (EF-1α) was amplified and sequenced using EF-1 and EF-2 primers, as described by O'Donnell et al. (1998). At least one strain from each cluster and subcluster obtained by ISSR-PCR was selected randomly for sequencing. Amplifications were performed in a 50 μl final volume containing 15–20 ng of genomic DNA, 1X reaction buffer, 0.3 μM of each primer, 0.2 mM dNTPs, 1.5 mM of $MgCl_2$, 1.5 U Taq DNA polymerase (Invitrogen, Buenos Aires, Argentina). A negative control, containing all reagents and primers but no fungal DNA, was included. Amplification was conducted in a PTC-2000 Thermal Cycler. The PCR conditions were: 94 °C, 2 min then 35 cycles of 94 °C, 30 s, 54 °C, 40 s, 72 °C, 50 s, followed by a final extension step of 5 min, 72 °C. All the PCR products generated were resolved on 1.5% agarose gels. The gels were stained with ethidium bromide (5 μg/ml) and visualized under UV light. After amplification, the products were purified using DNA purification columns (DNA Wizard DNA Clean-Up Kit, Promega, Madison, WI). Both DNA strands were sequenced using an ABI 3730 sequencer (Applied Biosystems). Sequencing errors were detected and corrected using BioEdit sofware version 7.0.9.0

(Thompson et al. 1994). Nucleotide sequence comparisons were performed using the Basic Local Alignment Search Tool (BLAST) network services of the National Centre for Biotechnology Information (NCBI) database. Multiple sequence alignment of the EF-1α gene was performed using the Web-based program MAFFT (Katoh and Standley 2013). EF-1α sequences from reference FGSC strains and other *Fusarium* species obtained from GenBank were included in the analysis. Based on this alignment, a phylogenetic analysis was performed by maximum parsimony method using TNT 1.1 (Goloboff et al. 2008). The robustness of the tree obtained was evaluated by 1000 bootstrap replications. *Fusarium pseudograminearum* 11,436 was used as the outgroup.

Trichothecene genotype determination

Multiplex PCR experiments were conducted with 10–25 ng of fungal DNA in a total volume of 50 μl of 1X reaction buffer containing 1.5 mM $MgCl_2$, 2 U Taq DNA polymerase (Invitrogen, Buenos Aires, Argentina), 0.2 mM dNTPs, 0.2 mM each of the Tri3 primers (Tri3F971, Tri3F1325 and Tri3R1679), and 0.1 mM each of primers Tri7F340, Tri7R965, 3551H and 4056H (Quarta et al. 2006). A negative control, containing all reagents and primers but no fungal DNA, was included with every set of reactions.

PCR was conducted in a PTC-2000 Thermal Cycler. The PCR conditions were: 94 °C, 3 min then 35 cycles of 94 °C, 30 s, 53 °C, 30 s, 72 °C, 1 min, followed by a final extension step of 10 min, 72 °C. All the PCR products generated were resolved on 1.5% agarose gels. The gels were stained with ethidium bromide (5 μg/ml) and visualized under UV light. DNA from strains with known NIV, 3ADON and 15ADON chemotypes were used as positive controls.

DON production in vitro

Fusarium strains were cultured in Erlenmeyer flasks (250 ml) containing 25 g of long grain rice and 10 ml of distilled water. Flasks were autoclaved for 30 min at 121 °C, allowed to cool at room temperature for at least 24 h, and then autoclaved a second time. Each flask was inoculated with a 3 mm diameter agar disk taken from the margin of a colony actively growing on synthetic nutrient agar (SNA) at 25 °C for seven days (Leslie and Summerell 2006). Flasks were shaken by hand daily for

1 week to disperse the fungus throughout the rice. These cultures were incubated for 28 days at 25 °C in dark. At the end of the incubation period, the contents of the flask were dried at 50 °C for 24 h and then stored at −20 °C until analyzed for toxin.

Toxin analyses were made as described by Cooney et al. (2001), with modifications. In particular, each sample was finely ground in a laboratory grinder and then homogenized. A sub-sample of ground rice (15 g) was extracted by mixing with 40 ml acetonitrile/ methanol (14:1, v/v), shaken for 2 h on an oscillatory shaker (150 rpm), and then filtered through Whatman No. 1 filter paper. A syringe (3 ml capacity) plugged with glass wool and dry-packed with alumina/carbon (20:1, w/w; 500 mg) was used as a mini-cleanup column. A 2 ml aliquot of extract was applied to the column, allowed to drain under gravity, and the eluent collected. The column was washed with 500 μl aceto-nitrile/methanol/water (80:5:15, v/v), and the combined eluents evaporated to dryness under N_2 at 50 °C. The cleaned-up residue was dissolved in 500 μl methanol/ water (5:95, v/v).

The HPLC system consisted of a Hewlett Packard model 1100 pump (Palo Alto, CA) connected to a Hewlett Packard 1100 Series variable wavelength detector and a data module Hewlett Packard Kayak XA (HP ChemStation Rev. A.06.01). Chromatographic separations were performed on a Luna™ C18 reversed-phase column (100 × 4.6 mm, 5 μm particle size) connected to a guard column, SecurityGuard™(4 × 3.0 mm), filled with the same phase. The mobile phase consisted of methanol/water (12:88, v/v), at a flow rate of 1.5 ml/min. The detector was set at 220 nm with an attenuation of 0.01 AUFS. The injection volume was 50 μl. Retention times for NIV and DON were 400 and 900 s, respectively. Quantification was relative to external standards of DON and NIV (Sigma-Aldrich Co. St Louis,MO) of 1 to 4 μg/ml in methanol/water (5:95). The quantification limit for each toxin was 50 μg/kg.

Results

Strains isolation

Sixty-two putative *F. graminearum* strains were isolated from five out of the seven localities analyzed. Six strains from Balcarce, 12 from Miramar, 15 from Pieres (southeast area), 5 from Barrow and 24 from La Dulce (mid-south area). None were isolated from Cabildo and Bordenave (southwest area) (Table 1 and Table 3).

ISSR analysis

Four out of 15 ISSR primers (ISSR6, ISSR7, ISSR8 and ISSR10) were selected based on their ability to consistently amplify the same fragments from a given strain; reproducibility of amplified bands was confirmed at least with two repetitions. A total of 78 fragments between 200 and 2500 bp were amplified and among them 72 (92%) were polymorphic. An example of an amplification reaction with primer ISSR6 is presented in Fig. 2.

The combined ISSR data of the strains were subjected to cluster analysis using Nei and Li's coefficient with the UPGMA clustering method. The cluster analysis of ISSR data revealed two distinct clusters (I and II) with 97% bootstrap support values amongst 62 strains morphologically identified as *F. graminearum* (Fig. 3). Cluster I (85% bootstrap) contained strains belonging to the FGSC whereas cluster II (57% bootstrap) grouped strains of *F. cerealis* and the *F. culmorum* reference strain. Seventy-eight percent of the strains isolated in this study clustered together with the *F. graminearum* reference strain while 22% clustered together with *F. cerealis* reference strain (78% bootstrap support value).

Only three pairs of strains displayed identical ISSR pattern. The CCC showed a very good fit between the cophenetic matrix and the matrix upon which the clustering was based, with $r = 0.95$. The AMOVA (fixation index of differentiation between population divisions $\Phi ST = 0.178$; $P < 0.001$) indicated that most of the variation resulted from genetic differences within (82%), rather than from differences between the southeast and mid-south populations (18%).

Representative strains of each cluster and subcluster were subjected to sequencing of EF-1α gene and were compared to sequences from the Genbank for results verification. Sequence analysis by the BLAST tool against other sequences from reference strains revealed that 10 strains (RCFG6053, RCFG6011, RCFG6031, RCFG6044, RCFG6048, RCFG6030, RCFG6001, RCFG6069, RCFG6021, and RCFG6058) had high homology (99%) to *F. graminearum* and seven strains (RCFG6018, RCFG6032, RCFG6029, RCFG6017, RCFG6024, RCFG6045 and RCFG6066) to *F. cerealis* (97 to 99% homology). The results support

Table 3 Species, origin, harvest year and trichothecene genotype of *Fusarium* strains isolated from durum wheat in Argentina

Strain	Species	Origin	Harvest year	Trichothecene genotype	EF-1α (Accession no)
RCFG6000	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6001	*F. graminearum*	La Dulce	2008	15-ADON	KX359399
RCFG6002	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6003	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6004	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6005	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6006	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6007	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6008	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6009	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6010	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6011	*F. graminearum*	La Dulce	2008	15-ADON	KX359393
RCFG6012	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6013	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6014	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6015	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6016	*F. graminearum*	La Dulce	2008	15-ADON	
RCFG6017	*F. cerealis*	La Dulce	2008	NIV	KX359405
RCFG6018	*F. cerealis*	Miramar	2009	NIV	KX359402
RCFG6019	*F. graminearum*	Miramar	2009	15-ADON	
RCFG6020	*F. cerealis*	Miramar	2009	NIV	
RCFG6021	*F. graminearum*	Miramar	2009	15-ADON	KX359401
RCFG6022	*F. cerealis*	La Dulce	2009	NIV	
RCFG6023	*F. cerealis*	La Dulce	2009	NIV	
RCFG6024	*F. cerealis*	Miramar	2009	NIV	KX359406
RCFG6025	*F. graminearum*	Pieres	2010	15-ADON	
RCFG6026	*F. graminearum*	Pieres	2010	15-ADON	
RCFG6027	*F. graminearum*	Pieres	2010	15-ADON	
RCFG6028	*F. graminearum*	Pieres	2010	15-ADON	
RCFG6029	*F. cerealis*	Pieres	2010	NIV	KX359404
RCFG6030	*F. graminearum*	Pieres	2010	15-ADON	KX359397
RCFG6031	*F. graminearum*	Pieres	2010	15-ADON	KX359394
RCFG6032	*F. cerealis*	Pieres	2010	NIV	KX359403
RCFG6033	*F. cerealis*	Pieres	2010	NIV	
RCFG6034	*F. graminearum*	Pieres	2010	15-ADON	
RCFG6035	*F. graminearum*	Pieres	2010	15-ADON	
RCFG6036	*F. graminearum*	Pieres	2010	15-ADON	
RCFG6041	*F. cerealis*	Pieres	2010	NIV	
RCFG6042	*F. cerealis*	Pieres	2010	NIV	
RCFG6043	*F. cerealis*	Pieres	2010	NIV	
RCFG6044	*F. graminearum*	Barrow	2010	15-ADON	KX359395
RCFG6045	*F. cerealis*	Barrow	2010	NIV	KX359407
RCFG6047	*F. graminearum*	La Dulce	2010	15-ADON	
RCFG6048	*F. graminearum*	Miramar	2010	15-ADON	KX359396

Table 3 (continued)

| Strain	Species	Origin	Harvest year	Trichothecene genotype	EF-1α (Accession no)
RCFG6049	*F. graminearum*	Miramar	2010	15-ADON	
RCFG6050	*F. graminearum*	Miramar	2010	15-ADON	
RCFG6051	*F. graminearum*	Miramar	2010	15-ADON	
RCFG6052	*F. graminearum*	Balcarce	2010	15-ADON	
RCFG6053	*F. graminearum*	Barrow	2010	15-ADON	KX359392
RCFG6054	*F. graminearum*	Balcarce	2010	15-ADON	
RCFG6055	*F. graminearum*	Balcarce	2010	15-ADON	
RCFG6056	*F. graminearum*	Miramar	2010	15-ADON	
RCFG6058	*F. graminearum*	La Dulce	2010	15-ADON	KX359398
RCFG6059	*F. graminearum*	Balcarce	2010	15-ADON	
RCFG6060	*F. graminearum*	La Dulce	2010	15-ADON	
RCFG6064	*F. graminearum*	Miramar	2010	15-ADON	
RCFG6065	*F. graminearum*	La Dulce	2010	15-ADON	
RCFG6066	*F. cerealis*	Barrow	2012	NIV	KX372274
RCFG6067	*F. graminearum*	Barrow	2012	15-ADON	
RCFG6068	*F. graminearum*	Balcarce	2012	15-ADON	
RCFG6069	*F. graminearum*	Balcarce	2012	15-ADON	KX359400
RCFG6070	*F. graminearum*	Cabildo	2012	15-ADON	
RCFG6071	*F. graminearum*	Miramar	2012	15-ADON	

15-ADON: 15-acetyldeoxynivalenol; NIV: nivalenol

the characterization of the strains deduced from the ISSR analysis.

Further, a phylogenetic analysis was performed with the sequences obtained together with sequences of other *Fusarium* species retrieved from the *Fusarium* ID (Fig. 4). Strains RCFG6053, RCFG6011, RCFG6031, RCFG6044, RCFG6048, RCFG6030, RCFG6001, RCFG6069, RCFG6021 and RCFG6058 formed a distinct clade with *F. graminearum* reference strain (100% bootstrap) that was distinct from the other FGSC species included in the analysis and strains RCFG6018, RCFG6032, RCFG6029, RCFG6017, RCFG6024, RCFG6045 and RCFG6066 form a clade with *F. cerealis* reference strain (100% bootstrap). These

Fig. 2 Amplification patterns of *Fusarium* strains with primer ISSR6. Lanes 1: RCFG6000; 2: RCFG6002; 3: RCFG6004; 4: RCFG6006; 5: RCFG6008; 6: RCFG6009; 7: RCFG6011; 8: RCFG6013; 9: RCFG6014; 10: RCFG6015; 11: *F. graminearum* NRRL 31084; 12: Molecular size marker (100 bp DNA Ladder Marker Invitrogen); 13: RCFG6017; 14: RCFG6019; 15: RCFG6020; 16: RCFG6022; 17: RCFG6023; 18: RCFG6024
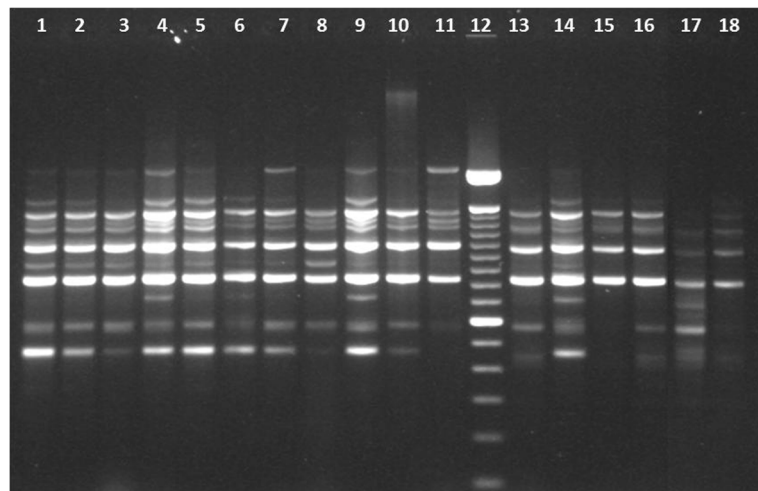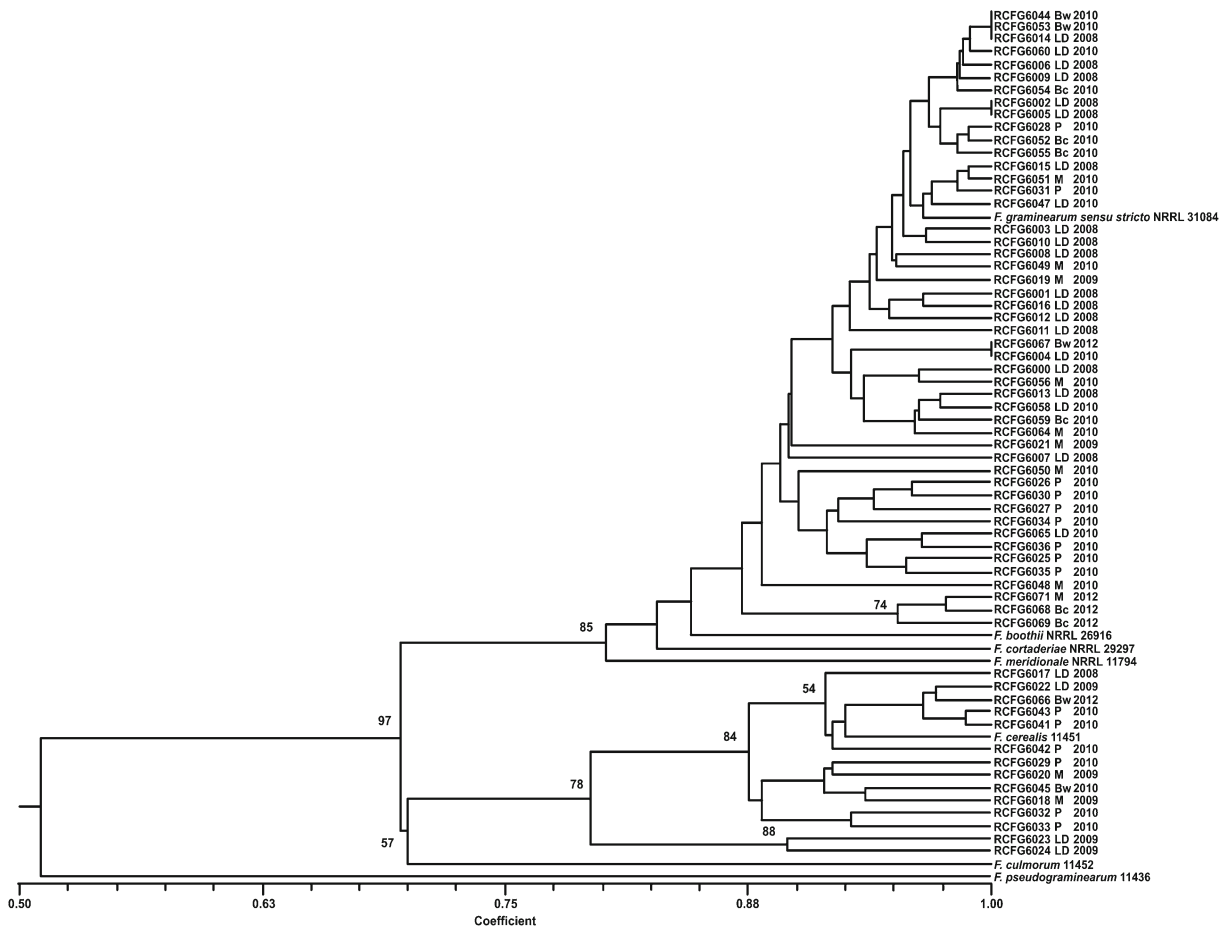
Fig. 3 Cluster analysis dendrogram of the 62 *Fusarium* strains using ISSR data. Numbers above branches are bootstrap values. Only values above 50% are indicated. *Fusarium pseudograminearum* 11,436 was used as the outgroup. Abbreviations used for localities: Bw: Barrow, Bc: Balcarce, LD: La Dulce, M: Miramar, P: Pieres. Harvest year is indicated next to each strain

results confirmed the identity of the majority of the strains isolated from durum wheat as *F. graminearum* as well as they suggest that durum wheat grains were also contaminated with *F. cerealis*.

Trichothecene genotype

The multiplex PCR assay showed that all *F. graminearum* strains amplified two fragments of 525 and 708 bp corresponding to the 15ADON genotype, the NIV and 3ADON genotypes were not detected among them, while all *F. cerealis* strains showed the NIV genotype since amplification of one fragment of 625 bp was observed (Table 3).

Mycotoxin production by Fusarium strains

Fusarium graminearum strains analyzed produced only DON in levels ranging between 4.56 and 2383.5 mg/kg (mean = 330.16 mg/kg) whereas *F. cerealis* strains produced NIV in levels ranging from nd to 26.56 mg/kg (mean = 5.56 mg/kg), there were two strains that did not produce the toxin.

Discussion

Sixty-two strains identified morphologically as *F. graminearum* were isolated from five out of seven localities analyzed. Cabildo and Bordenave, the two localities where no strains were isolated, are situated in the southwest of Buenos Aires province (southwest area). This area has climatic conditions unfavorable for FHB development as was showed by Moschini and Bischoff (2007) in a study of climate risk of the Pampas region regarding FHB, the authors showed that FHB gradually decreases towards this area due to its semi-arid climate.
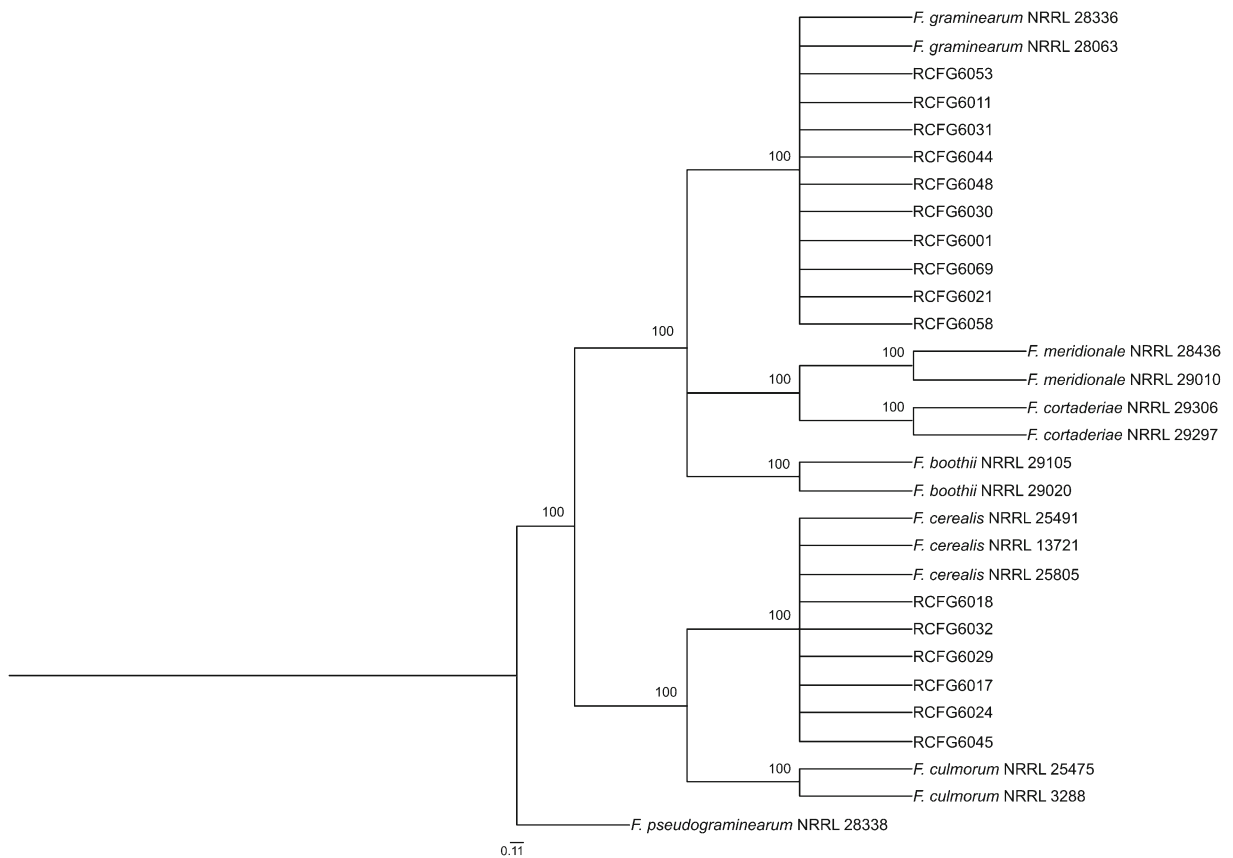
Fig. 4 Tree inferred from EF-1α gene partial sequences. Branches that received ≥50% maximum parsimony (MP) bootstrap values are indicated above branches. *Fusarium pseudograminearum* strain NRRL 28338 was used as the outgroup

The ISSR results demonstrate that among the strains isolated and identified morphologically as *F. graminearum*, there were 14 strains that were molecularly identified as *F. cerealis*. *Fusarium cerealis* (synonym *F. crookwellense*) is often associated with ear rot of maize and it is considered a secondary pathogen in the FHB of wheat (Bottalico and Perrone 2002; Logrieco et al. 2003). Several authors have reported the isolation of this species from symptomatic wheat spikes but at low frequencies (Schmale et al. 2011; Tan et al. 2004; Zhang et al. 2012). However, this fungus was considered one of the most aggressive pathogen toward wheat during a greenhouse assay along with *F. graminearum* and *F. culmorum* (Xue and Armstrong 2004). In 2008, one of the most severe FHB epidemic years in China, 15 *Fusarium* strains were isolated from barley and eight strains were isolated from wheat spikes. All 15 strains from barley and six of the eight strains from wheat were identified

as *F. cerealis*, being this the first report of *F. cerealis* causing FHB on barley and wheat in China (Zhang et al. 2011). Also, in Argentina, this species has been associated to FHB on barley (Castañares et al. 2013). More recently, Amarasinghe et al. (2014) reported for the first time in Canada the isolation of this pathogen from naturally infected wheat varieties. In addition, they carried out pathogenicity tests that revealed that *F. cerealis* was able to cause FHB in this cereal but was less aggressive than *F. graminearum* and also it was able to produce NIV on the infected plants. *Fusarium graminearum* could be misidentified easily with *F. cerealis*, since both species are very similar morphologically, their macroconidia have similar shape, they lack microconidia and they are difficult to distinguish in commonly used media like PDA (Gagkaeva 2010; Leslie and Summerell 2006).

In addition, the trichothecene genotype of the strains isolated was determined by multiplex PCR assay and the capability to produce the toxins was

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assayed on rice. All *F. graminearum* strains presented DON/15ADON genotype and were able to produce DON in a wide range of levels. Similar results were obtained in previous studies on the toxigenic potential of *F. graminearum* strains isolated from wheat in South America (Astolfi et al. 2012; Pan et al. 2013). In Argentina, previous studies also found that the predominant genotype among *F. graminearum* strains was DON/15ADON but they also found isolates with the DON/3ADON and DON/NIV genotypes. Regarding the DON production, the levels here detected are in accordance with those found in these studies (Alvarez et al. 2009; Reynoso et al. 2011; Ortega et al. 2016). To determine the trichothecene genotype of *F. graminearum* populations isolated from wheat is important since it has been seen that those with the 3ADON genotype were more aggressive than the 15ADON genotype in spring wheat, and also the strains with the 3ADON genotype were capable of producing higher amounts of DON (Puri and Zhong 2010; von der Ohe et al. 2010; Yli-Mattila and Gagkaeva 2010). *Fusarium cerealis* strains presented the NIV genotype and most of them produced the toxin on rice. Amarasinghe et al. (2014) evaluated the toxin production of *F. cerealis* strains in wheat plants and found NIV levels ranging from 0.66 to 6.15 mg/kg which are in accordance with the levels detected in the present study.

On the other hand, ISSR markers proved to be an efficient tool to assess genetic variability of the strains and also to discriminate *Fusarium* species causing FHB. The ISSR study revealed that durum wheat grains were mostly contaminated by *F. graminearum* since the 77% of the strains isolated clustered together with the *F. graminearum* reference strain. This result provides further evidence that *F. graminearum* is the predominant species of the FGSC isolated from durum wheat in Argentina. Furthermore, the genetic variability detected within the two populations was considerably higher than between populations. The results are in agreement with previous studies carried out in North America (Schmale Iii et al. 2006; Zeller et al. 2003), France (Boutigny et al. 2013), Brazil (Astolfi et al. 2012) and Uruguay (Pan et al. 2016; Umpierrez-Failache et al. 2013). In Argentina, Ramirez et al. (2007) and Alvarez et al. (2011) found that the main species within the FGSC responsible of FHB of common wheat was

F. graminearum and also found a high level of genetic diversity within the populations. It is noteworthy that this is the only species of the FGSC found in wheat in our country so far since other members of this complex have been isolated from other crops such as maize and soybean (Barros et al. 2012; Sampietro et al. 2011). In this sense, it could be hypothesized that it exists a host preference within the FGSC in Argentina as it was suggested by Boutigny et al. (2011) in South Africa. However, recently, others species within this complex such us *F. meridionale*, *F. cortaderiae*, *F. asiaticum*, *F. brasilicum* and *F. austroamericanum* have been reported in wheat in Brazil and Uruguay (Astolfi et al. 2012; Umpierrez-Failache et al. 2013).

The present study evaluated the *Fusarium* species present in durum wheat grains from the south of Buenos Aires province, the main growing area of this cereal in Argentina. The main species isolated from the samples was *F. graminearum*, however it was also detected the presence of *F. cerealis*. This finding is of great importance since recently this fungus has been associated to FHB of barley in Argentina (Castañares et al. 2013) where the main growing area is also located at the south of Buenos Aires province. Also, in our laboratory it has been detected NIV in samples from durum wheat grains obtained from this region in levels ranging from 2830 to 8190 µg/kg (data not publish). Considering that *F. graminearum* strains here isolated were DON/15ADON genotype, it could be probable that the NIV contamination of this cereal was due to *F. cerealis*. Therefore, we consider important to continue investigating if this fungus contribute to FHB of wheat in this region and to the contamination with the mycotoxin since NIV is consider to be more toxic to animals than DON (Ueno 1977; Visconti et al. 1991).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal studies This article does not contain any studies with human or animal subjects.

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