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Trichothecene genotypes and production profiles of *Fusarium graminearum* isolates obtained from barley cultivated in Argentina

Eliana Castañares [a], Diana Ramirez Albuquerque [b], María Inés Dinolfo [a], Virginia Fernandez Pinto [b], Andrea Patriarca [b], Sebastián Alberto Stenglein [a,c,*]

[a] Laboratorio de Biología Funcional y Biotecnología (BIOLAB)-CICBA-INBIOTEC, Facultad de Agronomía de Azul-UNCPBA, Av. República de Italia 780, 7300 Azul, Buenos Aires, Argentina
[b] Laboratorio de Microbiología de Alimentos, PROPLAME-PRHIDEB, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, UBA, C1428EGA Buenos Aires, Argentina
[c] Cátedra de Microbiología, Facultad de Agronomía de Azul-UNCPBA, Av. República de Italia 780, 7300, Azul, Buenos Aires, Argentina

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ABSTRACT

Fusarium graminearum is one of the most important pathogens isolated from small cereal grains with Fusarium Head Blight symptoms. The presence of this fungus is often linked to the occurrence of several mycotoxins in barley and wheat. The aim of our study was to characterize trichothecene genotypes and production profiles of *F. graminearum sensu stricto* isolates obtained from barley grains in Argentina. A total of 110 *F. graminearum s.s.* isolates were analyzed by PCR assays to predict deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON) and nivalenol (NIV) production, and all isolates were found to belong to the same molecular 15-ADON genotype. Trichothecene production in autoclaved rice was analyzed by using gas chromatography (GC) and confirmed by GC–MS. Of the 110 isolates, 95% were able to produce DON, 71% produced 15-ADON, 63% 3-ADON and 52% NIV. With the exception of a single isolate, all isolates that produced NIV, also produced DON. However, the NIV production was very low, ranging from 0.13 to 0.30 μg/g. Six different production profiles of DON and its acetyl-derivatives were detected, the predominant being simultaneous production of DON, 3-ADON and 15-ADON, followed by DON production, and DON and 15-ADON co-production. This work is the first attempt to characterize the trichothecene genotypes and production profiles of *F. graminearum s.s.* isolates from Argentinean barley.

1. Introduction

Barley (*Hordeum vulgare* L.) is the second main winter crop in Argentina, where its cropping area has increased to 1.5 million ha in the last growing season (2012–2013), with a production of ±5 million tons. The principal use of this crop is for malt production, although substandard grains are used for animal feed. *Fusarium graminearum* (syn. *Gibberella zeae*) is the principal causal agent of *Fusarium* Head Blight (FHB) on small cereal grains worldwide. Even though the main problem of the infection is yield loss, a cause of great concern is the ability of *F. graminearum* to contaminate the barley kernels with type B trichothecenes such as nivalenol (NIV) and its acetylated derivatives, and deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON) or 3-acetyldeoxynivalenol (3-ADON) (Desjardins, 2006). In general, DON is associated with vomiting, feed refusal and also quality loss of malt and beer gushing (Schwarz et al., 1996; Oliveira et al., 2012), whereas NIV is more toxic to humans and animals than DON (Minervini et al., 2004).

In recent years, several PCR assays have been developed to predict *F. graminearum*/*F. culmorum* chemotypes based on the sequences of the trichothecene biosynthesis pathway genes. For example, primers to differentiate DON from NIV producers based on sequence alleles at *Tri7* and *Tri13* were developed by Lee et al. (2001, 2002) and Chandler et al. (2003), primers based on *Tri5* and *Tri6* gene sequences were developed to differentiate high or low DON producers (Bakan et al., 2002); *Tri3*, *Tri5*, and *Tri7* gene sequences were used to design primers used to differentiate 15-ADON, 3-ADON and NIV producers, respectively (Quarta et al., 2006). Three different chemotypes have been described in *F. graminearum* corresponding to different trichothecene profiles: NIV chemotype, when NIV is produced; 15-ADON chemotype, when DON and 15-ADON are produced; 3-ADON chemotype, when DON and 3-ADON are produced (Desjardins, 2006). On the other hand, such classification should be correctly referred to genotypes since it is based on DNA sequences (Desjardins, 2008). Trichothecene chemotype definition should only be used when the chemical phenotype is expressed and detected by chemical analyses, because the detection of a given trichothecene genotype does not always predict the presence

* Corresponding author at: Laboratorio de Biología Funcional y Biotecnología (BIOLAB)-CICBA-INBIOTEC. Facultad de Agronomía de Azul-UNCPBA. Av. República de Italia 780, 7300, Azul, Buenos Aires, Argentina. Tel./fax: +54 2281 433291.
E-mail address: stenglein@faa.unicen.edu.ar (S.A. Stenglein).

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of the corresponding metabolites (Desjardins, 2008). Genotyping results are usually well in accordance with chemotypes (e.g. Ward et al., 2002; Quarta et al., 2006; Yli-Mattila et al., 2009; Reynoso et al., 2011).

The toxigenic potential of *F. graminearum sensu stricto* strains varies around the world. In France, Germany, Italy, Luxembourg, and Turkey, 15-ADON producers were higher in frequency than those of NIV or 3-ADON (Pasquali et al., 2010; Mugrabi de Kuppler et al., 2011; Yörük and Albayrak, 2012; Boutigny et al., 2014; Somma et al., 2014). However, studies made in different regions of Russia and adjacent countries showed that 3-ADON producing isolates of *F. graminearum* are dominant in northern Europe. The 3-ADON chemotype was found to be prevalent in Scandinavia, Finland and north-western Russia, whereas the 15-ADON chemotype was more common in southern Europe and China. Both 3-ADON and 15-ADON chemotypes are common in the Russian Far East (Jestoi et al., 2008; Yli-Mattila et al., 2009; Yli-Mattila, 2010). *F. graminearum* isolates with the 15-ADON genotype were found in previous studies as largely responsible for FHB in North America. More recent studies indicated localized heterogeneity among *F. graminearum* populations (Ward et al., 2008). Gale et al. (2011) observed that NIV, 15-ADON and 3-ADON genotype populations obtained from wheat varied according to the geographic origin in the USA. In South America (Uruguay and Brazil) *F. graminearum* isolates of the 15-ADON genotype were reported to predominate in wheat (Scoz et al., 2009; Pan et al., 2013).

In Argentina *F. graminearum s.s.* studies regarding the genotype and/or the production of trichothecenes NIV, DON, 15-ADON, 3-ADON have focused on wheat. Reynoso et al. (2011) using a multiplex PCR assay found that the 92% of the *F. graminearum* isolates evaluated had the 15-ADON genotype. Fernandez Pinto et al. (2008), Alvarez et al. (2009) and Reynoso et al. (2011) revealed that the most common profile of trichothecene production was DON + 15-ADON, although 3-ADON and NIV producers were also detected.

Genotype and chemical profile surveys of *F. graminearum s.s.* isolated from barley have not been previously carried out in Argentina. There have been few studies analyzing *F. graminearum* isolates obtained from barley around the world. Yang et al. (2008) found that most of the isolates from Chinese barley belonged to the DON molecular chemotype (genotype). Astolfi et al. (2011) and Boutigny et al. (2014) reported that 15-ADON genotype was predominant in barley from Southern Brazil and France, respectively.

The aim of our study was to characterize trichothecene genotypes and chemical profiles of *F. graminearum s.s.* isolates obtained from barley grains from different fields in the main production region of Argentina.

2. Materials and methods

2.1. Fungal isolation

F. graminearum isolates were obtained from different barley grain samples collected from commercial fields of Argentina during the 2010, 2011 and 2012 growing seasons (Table 1). Grain samples (200 g) were reduced to 400 grains with a grain divider, surface-disinfected, placed onto potato dextrose agar (PDA) with 0.25 g chloramphenicol/L and incubated for 4–7 days at 25 ± 2 ºC under a 12 h light/dark cycle. Single spore *F. graminearum* isolates were morphologically identified on PDA and on Spezieller Nährstoffarmer Agar (SNA) according to Leslie and Summerell (2006).

2.2. DNA isolation

Genomic DNA from a total of 110 monosporic *F. graminearum* isolates (Table 1) was extracted using the cetyltrimethylamonium bromide (CTAB) method according to Stenglein and Balatti (2006). The quality of fungal DNA was examined by electrophoresis in 0.8% (w/v) agarose gels containing GelRed™ (Biotium, Hayward, USA) at 80 V in 1× Trisborate-EDTA buffer for 3 h at room temperature. The DNA was visualized under UV light. The DNA concentration was estimated with a fluorometer (Qubit™-Invitrogen, Buenos Aires, Argentina).

2.3. Polymerase chain reaction assays

2.3.1. Fusarium graminearum sensu stricto identification

To confirm morphological identifications a *F. graminearum*-specific PCR was performed for the 110 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. *F. graminearum* gives a product of about 400 bp, while *F. asiaticum* (NRRL 13818, used as control) gives a PCR product of about 550 bp and *F. meridionale* (NRRL 28436, used as control) gives a product of about 500 bp.

PCR assays were carried out using 10–20 ng of DNA in a total volume of 25 μL containing 10× reaction buffer, 0.5 μM of each primer, 200 μM of each dNTP (Genbiotech S.R.L.), 2.5 mM $MgCl_2$, and 1.25 U of *Taq* DNA polymerase (Inbio-Highway, Tandil, Argentina). DNA amplifications were performed in a XP thermal cycler (Bioer Technology Co.).

2.3.2. Genotype determination

PCR-trichothecene genotypes were classified into NIV or DON with primers Tri13NIVF/Tri13R and Tri13DONF/Tri13R, respectively, according to Chandler et al. (2003). NIV, DON, 15-ADON, and 3-ADON were also determined by a multiplex-PCR for all isolates with primers Tri7F340/Tri7R965, 3551H/4056H, Tri3F971/Tri3R1679 and Tri3F1325/Tri3R1679, respectively (Quarta et al., 2006).

F. graminearum Cv1.2 (15-ADON genotype), *F. graminearum* Cv12-C (3-ADON), and *F. meridionale* Cv811.1 (NIV) were used as DNA positive controls for trichothecene genotypes (Astolfi et al., 2011). The DNA of the isolates was kindly provided by PhD. Del Ponte, Departamento de Agronomia, Universidade Estadual de Maringá, Maringá, Brazil.

PCR products were examined by electrophoresis in 1.5% (w/v) agarose gels containing GelRed™ (Biotium, Hayward, USA) at 80 V in 1× Trisborate-EDTA buffer for 2 h. Fragments were visualized under UV light. Each PCR reaction was performed at least twice, running positive controls, plus the DNA ladder (100 bp) and the negative control in one gel, simultaneously.

2.4. Trichothecene analysis

Toxin analysis was performed according to Alvarez et al. (2009). Briefly, 250-mL Erlenmeyer flasks containing 25 g of sterilized rice and 15 mL of sterile distilled water were inoculated with each monosporic isolate. To determine the absence of trichothecenes in the rice, negative controls (triplicate) were prepared in the same way without inoculation. The inoculated flasks as well as the controls were incubated at 25 °C for 14 days and at 10 °C for 14 days in the dark.

Trichothecenes were extracted for 1 h at 300 rpm with 125 mL of acetonitrile:acetylacetate:water (50:41:9). The clean-up was performed with a column packed with charcoal:alumina:celite (0.7:0.5:0.3) and dried in Rotavap®. Gas chromatography, with ^{63}Ni electron capture detection Shimadzu Model GC17 equipped with split/splitless injector and fitted with RX-5MS capillary column (25 m × 0.2 mm id), were used to detect and quantify trichothecenes. The detection limits were 0.02 μg g^{-1} for DON and its acetyl derivatives, and 0.05 μg/g for NIV. Standards of DON, 15-ADON, 3-ADON and NIV were purchased from SIGMA Chemical Company (St Louis, MO, USA).

The presence of compounds was confirmed by Gas Chromatography–Mass spectrometer system (GC–MS QP 5050A, Shimadzu®) with Electron Impact (EI) mode (70 eV) as described by Alvarez et al. (2009).