



SHORT COMMUNICATION

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Presence of *Fusarium graminearum* sensu stricto associated with triticale (\times *Triticosecale* Wittmack) in Argentina

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Abstract

Aim of study: To report the occurrence of *Fusarium graminearum* sensu stricto (s.s.) on triticale grains from field samples in Argentina and the potential mycotoxin production for these isolates.

Area of study: Buenos Aires province, Argentina.

Material and methods: A total of 40 samples from different crops (barley, rye, triticale, and wheat) showing *Fusarium* head blight symptoms were taken during 2017/2018 harvest season. Colonies with colour and mycelium similar to *Fusarium* were taken and were morphologically and molecularly identified. The potential to produce deoxynivalenol, 15-acetyl deoxynivalenol and zearalenones was determined. Also, the Koch's postulates were used to evaluate the pathogenic capacity of the *F. graminearum* s.s. isolates in triticale.

Main results: Two *Fusarium* isolates were identified morphologically as *F. graminearum*, which were confirmed molecularly by PCR using the specific Fg16 F/R primers pair and by sequencing *red* and *tri101* genes. The sequences obtained were compared with those available in the NCBI database using BLAST tools, showing 99-100% homology with those belonging to *F. graminearum* s.s. The results demonstrated that *F. graminearum* s.s. isolates were pathogenic when triticale spikes were inoculated by spraying under greenhouse conditions.

Research highlights: To our knowledge, this is the first time that the presence of *F. graminearum* s.s. is reported associated with triticale in Argentina.

Additional key words: To our knowledge, this is the first time that the presence of *F. graminearum* s.s. is reported associated with triticale in Argentina.

Authors' contributions: Collected data: MID, MM and SAS. Morphological and molecular identification: MID, MM and EC. Pathogenicity test: MID and MM. Drafting the manuscript: MID, MM and EC. Critical revision of the manuscript: MM, EC and SAS.

Citation: Dinolfo, MI; Martínez, M; Castañares, E; Stenglein, SA (2019). Short communication: Presence of *Fusarium graminearum* sensu stricto associated with triticale (\times *Triticosecale* Wittmack) in Argentina. Spanish Journal of Agricultural Research, Volume 18, Issue 1, e10SC02. <https://doi.org/10.5424/sjar/2020181-14796>

Received: 01 Mar 2019. **Accepted:** 20 Feb 2020.

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Funding agencies/Institutions	Project/Grant
FONCyT/PICT	0213/2015
Universidad Nacional del Centro de la Provincia de Buenos Aires	

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Triticale (\times *Triticosecale* Wittmack), developed years ago by plant breeders, is a cross between wheat (*Triticum* sp. L.) and rye [*Secale cereale* L. (M. Bieb)], that combines characteristics of their parental crops. For instance,

as regard to bread-making quality, the amount of protein and gluten strongly correlates with strength and elasticity of the dough in wheat. However, in triticale, this correlation is lower because part of the protein comes from rye lacking the capacity to form gluten (Skovmand *et al.*, 1984). Considering nutritional qualities, the main

triticale grain component is starch with similar content to its parent, but with a better balance composition of amino acids showing advantages over the remaining crops (Villegas *et al.*, 1980). Regarding physical characteristics and chemical composition of grains such as protein content, crude fiber, ether extract, free sugars, and ash, triticale is in general intermediate between wheat and rye (Peña, 2004). All these characteristics allow that triticale not only is used as animal feed but also for human food in the preparation of baking products, such as different types of bread, noodles, and soft-wheat type products (Oliete *et al.*, 2010).

The capacity of producing a high amount of biomass and grain yield and the ability to adapt to different soils and climatic conditions allow the expansion of triticale to different regions. Hence, this crop is sown in different countries. Poland is the major producer with a production of 5.31 million tons (MT) followed by Germany (2.32 MT), Belarus (1.61 MT), France (1.59 MT) and China (0.98 MT) (<http://www.fao.org/faostat/es/?#data/QC>). In Argentina, 499,300 ha were destined to triticale during the 2017/2018 harvest season with a production of 15,205 kg for human consumption and the remaining used for animal feed (<http://datosestimaciones.magyp.gob.ar/>). Last years, the global triticale production increased due to be a good source of protein and energy used mainly for animal feed.

Over a long time, triticale was considered more resistant to diseases than wheat and rye. Due to the expansion of the area sown and the cultivation time, the same pathogens present in its parents spread in this crop losing this acquired advantage (Arseniuk, 1996). Several diseases caused mainly by species belonging to *Alternaria*, *Bipolaris*, *Dreschlera*, and *Fusarium* genera have been reported in triticale (Arseniuk, 1996). When environmental conditions such as temperature and humidity are optimal for its occurrence, *Fusarium* head blight (FHB) is the prevalent disease in crops, which leads mainly to a reduction in yield and quality of grains. The most common pathogen able to cause this disease is *F. graminearum* s.s., whose presence has been reported in wheat, barley, and rye, among others. This species, together with at least other 15 species, is a member of the *Fusarium graminearum* species complex (FGSC), with *F. graminearum* s.s. being the most common species found in grasses (Aoki *et al.*, 2012). Outside of this complex, *F. avenaceum*, *F. culmorum*, and *F. poae* have been frequently isolated from plants with FHB symptoms (Reis & Carmona, 2002). Other species known as *F. pseudograminearum* is mainly responsible for *Fusarium* crown rot, a prevalent disease where dry climatic conditions and conservation-farming practices are prevalent. The presence of this pathogen may interfere with the water and nutrient transports

in the plants producing unfilled grains and plant death (Desmond *et al.*, 2006).

The presence of *Fusarium* spp. leads to mycotoxin production whenever the climatic conditions are optimal to allow that the pathogen produces these secondary metabolites. These mycotoxins have a different impact on consumers depending on the type of mycotoxin produced. Trichothecenes are the most important group of *Fusarium* mycotoxins strongly associated with chronic and fatal toxicoses of humans and animals being deoxynivalenol (DON) and nivalenol (NIV) the most prevalent in different substrates (Dejardins & Proctor, 2007). Zearalenones (ZEA) are other mycotoxins structurally different to trichothecenes and acutely less toxic, which have not been associated with any fatal mycotoxicoses. Each *Fusarium* species has the genetic potential to produce different mycotoxins, whose production will depend on the environmental conditions present during the pathogen development. A correct morphological and molecular characterization is needed to identify the causal agent of the disease and thus know what mycotoxin could be present if the temperature and humidity conditions are optimal for mycotoxin production. Therefore, the objective of this study was to report the occurrence of *F. graminearum* s.s. on triticale grains from field samples in Argentina and the potential mycotoxin production for these isolates.

Material and methods

Field description and fungal identification

Different crops including barley (*Hordeum vulgare* L.), rye, triticale, and wheat were sampled from different fields in 14 locations of Buenos Aires province, Argentina, during the 2017/2018 harvest season. A triticale sample with around 20% of visual FHB symptoms in its glumes was manually collected at the sampling point located at 37°2'7.08" S, 62° 14'2.76" W (Fig. 1). The symptomatic grains were surface-disinfected by using 1% sodium hypochlorite solution, 70% ethanol and rinsed twice in sterile distilled water. Finally, grains were introduced on Petri dishes (90 mm) containing 2% potato dextrose agar (Laboratorios Britania S.A, Argentina) and incubated at 25 ± 2 °C in 12 h dark/light conditions for six days. After this incubation time, two colonies with colour and mycelium similar to *Fusarium* were taken and monospore cultures were performed on Spezieller Nährstoffarmer Agar. Finally, these single spore isolates were transferred to Petri dishes containing carnation leaf agar as recommended for morphological *Fusarium* identification in the same conditions described above (Leslie & Summerell, 2006). A slide of spores was prepared and stained with Lactophenol Blue. The

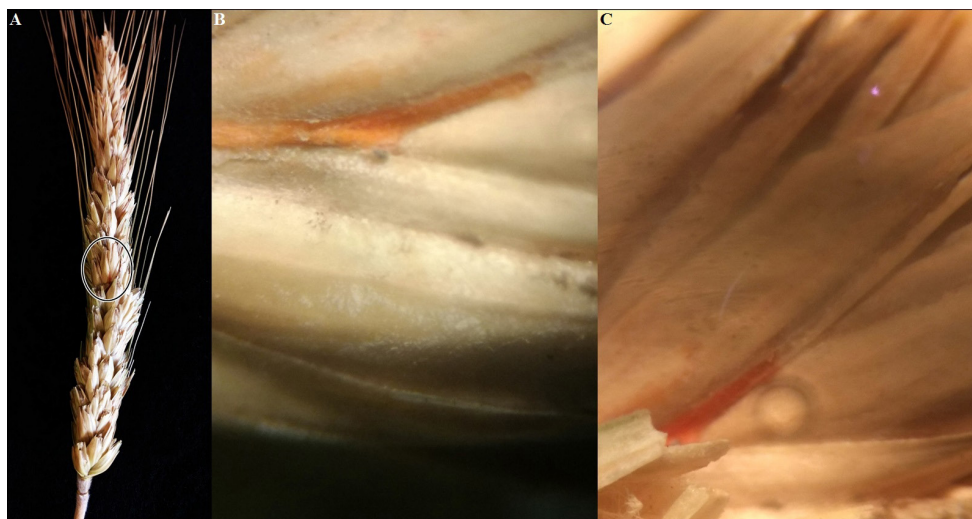


Figure 1. (A) Fusarium head blight (FHB) symptoms on triticale spikes (circle). (B-C) Presence of light pink/salmon coloured spores (sporodochia) on the glumes of individual spikelets.

fungus structures were observed and measured using an ocular micrometer disk in an optical microscope (Olympus CX31) at 40 magnification.

Molecular identification

Genomic DNA from two previously identified isolates (T1 and T2) was extracted based on the CTAB method (Stenglein & Balatti, 2006). These DNAs were used as templates in a *F. graminearum*-specific PCR using primers Fg16F and Fg16R with an annealing temperature of 62 °C according to Nicholson *et al.* (1998). Besides, PCR reactions were carried out using primers targeting portions of the reductase (*red*) and trichothecene 3-O-acetyltransferase (*tri101*) genes to identify the isolates to *F. graminearum* species level within the FGSC (O'Donnell *et al.*, 2000). The primer sequence for RED primers were: REDF (5' AGA CTC ATT CCA GCC AAG 3') and REDR (5' TCG TGT TGA AGA GTT TGG 3'), while for TRI101 were: TRI101F (5' CAA GAT ACA GCT CG CAC C 3') and TRI101R (5' CTG GGT AGT TGT TCG AGA 3') with an annealing temperature of 57°C and 62°C, respectively. All PCR reactions were performed using 10-20 ng of DNA in a total volume of 25 µL containing 10× reaction buffer, 0.5 µM of the respective primers, 200 µM of each dNTP (Inbio-Highway, Tandil, Argentina), 2.5 mM MgCl₂ and 1.25 U of *Taq* DNA polymerase (Inbio-Highway, Tandil, Argentina). PCR conditions were 95 °C for 2 min for an initial denaturalization, followed by 30 cycles consisting of 94 °C for 30 s, 30 s at temperature previously described for each primer pair, 72 °C for 5 min and finally an elongation step at 72 °C for 10 min performing in an XP Thermal cycler (Bioer Technology Co.). PCR products

were purified by using PureLink PCR purification kit (Invitrogen, Leohne, Germany) and were sequenced using Big Dye Termination version 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) in an Applied Biosystems Sequencer (ABI/Hitachi Genetic Analyzer 3130).

Potential mycotoxin production

PCR reactions were carried out using primers previously described for NIV and DON genotypes. The NIV, DON, and the two 3-acetyl deoxynivalenol (3-ADON) and 15-acetyl deoxynivalenol (15-ADON) genotypes were differentiated by using a multiplex PCR reaction with primers targeting portions of the *tri7* and *tri3* genes for NIV and DON genotypes, respectively with the same PCR conditions as described by Quarta *et al.* (2006). Primers based on *tri13* gene designed by Chandler *et al.* (2003) were equally tested. Finally, PKS4 primers developed by target the *PKS* gene needed to ZEA production were used according to Meng *et al.* (2010). DNA of a *F. graminearum* s.s. isolate (3.6) characterized as 15-ADON and ZEA genotypes, one 3-ADON *F. pseudograminearum* (14/11) and one NIV *F. meridionale* isolate (NRRL 28436) were used as controls for PCR reactions (Castañares *et al.*, 2016). NRRL isolates were kindly provided by the ARS Culture Collection. Products were examined by electrophoresis in 1.5% (w/v) agarose gels containing Gel-RedTM (Biotium, Hayward, CA, USA) at 80 V in 19 Trisborate-EDTA buffer at room temperature. Fragments were visualized under UV light. The size of the DNA fragments was estimated by comparing the DNA bands with a 100-bp DNA ladder (Genbiotech S.R.L.).

Gel images were photographed with a digital DOC 6490 system (Biodynamics S.R.L., Buenos Aires, Argentina).

Pathogenicity test

The pathogenic capacity of two isolates (T1 and T2) was tested by Koch's postulates. For this purpose, the inoculum was produced in a liquid medium containing carboxymethyl cellulose with uniform agitation (100 rpm, 25 ± 2 °C and darkness), while triticale plants were grown in a greenhouse in 5 L pots (one plant per pot). During flowering stage, 5 mL of spore suspension (10^5 conidia/mL) were sprayed in the spike until run off, while control pots were sprayed with sterile distilled water (Fig. 2A). The experiment design (inoculated and control treatment) was completely randomized with three replicates. Plants were covered with polyethylene bags for 96 h to ensure $>85\%$ of relative humidity and incubated in a greenhouse at 25 ± 2 °C. Spikes were photographed at 0, 2, 7, 14, 21 days post-inoculation (dpi) and at physiological maturity (Fig. 2). Finally, FHB symp-

toms were registered at 21 dpi. After physiological maturity, spikes from each treatment were threshed and cleaned manually. Symptomatic grains were surface-disinfected and the fungus was re-isolated and identified morphologically as *F. graminearum* as described above.

Results and discussion

Based on morphological characteristics, the isolates obtained from triticale were identified as *F. graminearum* (Schw.). The presence of sporodochia formed by macroconidia with 5-6 septa with a width average value of $4.5\text{-}5\ \mu\text{m}$ and a length average value of $50\text{-}60\ \mu\text{m}$ was visualized. Moreover, abundant chlamydospore presence were visualized, which are frequently found in the macroconidia, while microconidia were not found (Leslie & Summerell, 2006). The results of *F. graminearum* specific PCR amplified a fragment of 400-500 pb as expected according to Nicholson *et al.* (1998). To confirm molecularly our results, the final sequences of *red* and *tri101* fragments of T1 and T2 isolates were compared by



Figure 2. Fusarium head blight (FHB) progress in spikes inoculated with *F. graminearum* s.s. isolates collected on triticale. (A) Inoculation of the spike at anthesis. Disease progress at: 2 dpi (B), 7 dpi (C), 14 dpi (D), and 21 dpi (E). (F) grains at physiological maturity.

using BLAST tool of the NCBI. The sequences of T1 and T2 isolates of *red* fragments were submitted to GenBank under the following accession numbers: MH753697 and MH753698, respectively, showing 100% homology with KT334553.1, EF428773.1, and DQ925703.1. With regard to *tri101* fragments, these were submitted under MH753700 (T1) and MH753701 (T2) accession numbers showing 100% homology with KX774700.1, KX774428.1, and KX774497.1. The T1 and T2 isolates were deposited on BIOLAB fungal collection. The results of Koch's postulates confirmed that *F. graminearum* s.s. was able to infect triticale spikes being re-isolated from inoculated spikes. The symptoms were firstly observed at 2 dpi with mycelial growth on anthers (Fig. 2B). At 4 dpi, spikelets were completely affected (Fig. 2C), being the spike totally affected at 14 dpi (Fig. 2E). At physiological maturity, grains were severely affected showing mycelial growth and unfilled grains (Fig. 2F). Therefore, this is the first report that shows the presence of *F. graminearum* s.s. in triticale in Argentina. Although several works have been developed using different crops inoculated with *F. graminearum* (barley, oat, rye, triticale, and wheat), scarce information are available about the natural occurrence of *F. graminearum* s.s. in triticale (Logrieco *et al.*, 1990; Langevin *et al.*, 2004; Ferreira Geraldo *et al.*, 2006). Moreover, our results showed that both isolates amplified the 708 bp and 525 bp fragment corresponding to 15-ADON and DON genotypes, respectively, while no amplification was observed for 3-ADON and NIV genotypes (Fig. 3A). Confirming these results, the 282 bp fragment was also visualized by using the primers based on the *tri13* genes, while no products were observed for NIV genotypes (Fig. 3B). As regard to ZEA production, T1 and T2 showed a 280 bp fragment associated with the potential to produce this mycotoxin (Fig. 3C). The potential capacity of producing mycotoxins such as 15-ADON and ZEA could have a severe impact on consumer health. DON has been reported to be protein synthesis inhibitors targeting dividing cells such as leukocytes causing altered response immunity on consumers, while ZEA is responsible of pathological changes in the reproductive system (Chung *et al.*, 2003; Böhm *et al.*, 2010). In southern Brazil, the production of mycotoxins by *F. graminearum* in small cereals was evaluated by Ferreira Geraldo *et al.* (2006). In this study, seven isolates were isolated from triticale seeds and the DON and ZEA production *in vitro* was detected. The response of winter and spring triticale genotypes was evaluated against the natural source of *F. graminearum* inoculum at five sites across Canada (Veitch

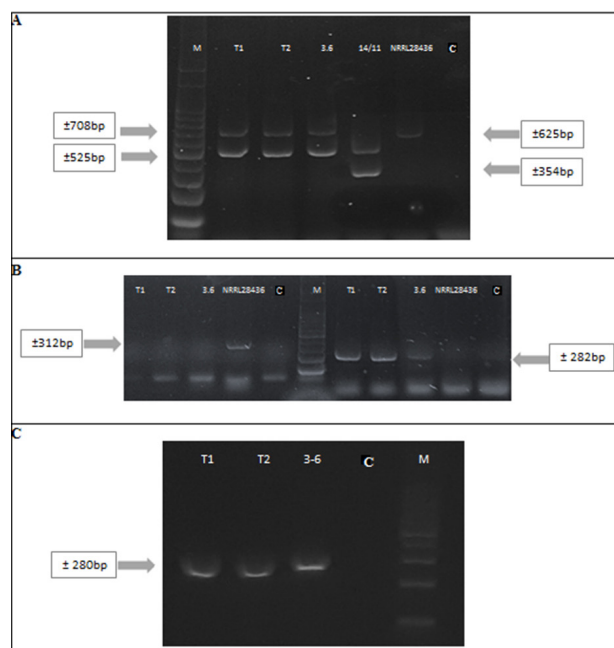


Figure 3. (A) Amplification of specific fragments by multiplex PCR: deoxynivalenol (DON), its acetylated derivatives (3-ADON and 15-ADON) and nivalenol (NIV). (B) Amplification of the DON and NIV specific fragments by using *tri13* genes. (C) Amplification of the ZEA specific fragments of *F. graminearum* s.s. M: molecular 100-bp marker (Genbiotech S.R.L.). T1-T2: *F. graminearum* s.s. from this study. 3-6: *F. graminearum* s.s. (15-ADON/ZEA controls). 14/11: *F. pseudograminearum* (3-ADON/ZEA control). NRRL28436: *F. meridionale* (NIV/ZEA controls). C: control with distilled water instead of DNA.

et al., 2008). The results showed a high susceptibility of both triticale genotypes to *Fusarium* with a considerable DON contamination. Future works are needed with the aim to evaluate the presence of pathogens in this promising crop to know its behavior and hence estimate what mycotoxins could be present in triticale grains.

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