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RAPID COMMUNICATION

Fusarium graminearum sensu stricto associated with head blight on rye (*Secale cereale* L.) in Argentina

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Abstract

Fusarium head blight (FHB) is one of the most important diseases that occurs in cereal regions worldwide and causes serious economic damage. This disease can be caused by several *Fusarium* species with *Fusarium graminearum* sensu stricto being the most common pathogen isolated from several crops. The aim of this study was to report the occurrence of *F. graminearum* sensu stricto on rye grains collected from field samples in Argentina and to determine the potential ability to produce deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA). Based on morphological characteristics, the isolate was identified as *F. graminearum* sensu stricto. To confirm molecularly, portions of the RED and TRI genes were sequenced and showed 99% similarity with the *F. graminearum* sensu stricto sequences available in the NCBI database. The potential to produce DON, 15-acetyldeoxynivalenol (15-ADON) and ZEA was determined. Moreover, Koch´s postulates were carried out. To our knowledge, this is the first report of *F. graminearum* sensu stricto associated with rye kernels in Argentina.

Keywords: deoxynivalenol, *Fusarium graminearum*, *Fusarium* head blight, nivalenol, rye, zearalenone

Rye (*Secale cereale* L.) is a highly versatile crop, being the second most used cereal, after wheat, in the production of bread. It is also an important cereal grain for production of mixed animal feeds. As a green plant, it is used as livestock pasture and as green manure in crop rotations; while as grain it is used for livestock feed and as feedstock in alcohol distilling, being used for the elaboration of whiskeys, vodkas, and in the brewing industry. Rye can be cultivated in areas that are generally not suitable for other cereal crops, because it is extremely winter hardy and can grow in sandy soils with low fertility. Currently, most of the rye produced worldwide is grown in cool temperate zones, but it can also be grown in semiarid regions and at high altitudes (Bushuk 2001). The total world production of rye for 2017/2018 harvest season was around 12.38 million tons, and the United States Department of Agriculture (USDA) estimate for 2018/2019 a production of 12.22 million tons. The main rye producers are the European Union (7.52 million tons), followed by Russia (2.54 million tons), Belarus (0.67 million tons) and Ukraine (0.51 million tons). During the 2017/2018 harvest season, USDA estimates around 45,000 hectares of production in Argentina, with a production of 90,000 tons positioning this country as the seventh world producer (USDA 2018). The main area of rye production in Argentina is distributed mainly in La Pampa (19,000 hectares), Buenos Aires (13,584 hectares) and Córdoba (8,300 hectares) provinces, representing 92% of the rye area of production for grain consumption in the country (MINAGRI 2018).

Fusarium head blight (FHB) is one of the most important diseases that occurs in cereals worldwide and causes serious economic damage. FHB is observed mainly in regions with a warm and wet climate coinciding with the flowering stage, thus affecting common winter crops such as wheat, barley and other small cereal grains (Dubin *et al.* 1997). This disease is caused by several *Fusarium* species which are able to reduce grain yield and produce different mycotoxins,

depending on the *Fusarium* species present. Mycotoxins can cause adverse effects in humans and animals through ingestion of contaminated cereal grains, being trichothecenes (inhibitors of eukariotic protein synthesis), one of the most important toxins produced by *Fusarium* species (Placinta *et al.* 1999; Bennett and Klich 2003). Deoxynivalenol (DON) and nivalenol (NIV) are the most common trichothecenes produced by *Fusarium* isolated from small grain cereals and several *Fusarium* species such as *F. graminearum*, *F. culmorum*, *F. poae* and *F. cerealis* are able to produce DON and/or NIV (Desjardins 2006). In addition, some *Fusarium* species can produce zearalenone (ZEA), a polyketide mycotoxin that has an oestrogenic effect on mammals affecting their reproductive systems (Desjardins and Proctor 2007).

Among *Fusarium* species causing FHB disease, *F. graminearum* sensu stricto (Schw.) (hereafter called *F. graminearum* s.s.) is the dominant species isolated worldwide, but other species such as *F. culmorum*, *F. avenaceum*, and *F. poae* are also frequently isolated (Reis and Carmona 2002; Stenglein *et al.* 2014). Some studies suggest that the diversity and distribution of *Fusarium* species depends on environmental variables, such as temperature and humidity conditions during the flowering stage, as well as agronomic practices such as crop rotation, tillage systems, and herbicide uses (Fernández *et al.* 2005; Xu *et al.* 2008). Although *F. graminearum* is the predominant FHB agent worldwide in winter crops, for example wheat and barley, other small cereal grains such as rye, triticale and oats are frequently infected by *Fusarium* species (Kiecana and Mielniczuk 2010; Góral *et al.* 2016; Schöneberg *et al.* 2018). As is known, *F. graminearum* has been reported to produce DON, NIV and ZEA (Marasas *et al.* 1984; Leslie and Summerell 2006). To our knowledge, there is no information available about the occurrence and distribution of *Fusarium* species in rye grains especially in Argentina. The aim of this study was to report the occurrence of *F. graminearum* s.s. on rye grains collected from field samples in Argentina. Also, the potential ability to produce DON, NIV and ZEA was tested using specific PCR assays, while morphological and molecular methods were carried out to confirm the presence of *F. graminearum* s.s. in rye grains.

During the 2017/2018 harvest season, a survey of fungi in winter crops (wheat, barley, triticale and rye) was carried out in different fields located in Buenos Aires province, Argentina. A total of 40 grain samples was manually collected from 14 different locations. The samples were taken from the main producing area of winter crops, mainly in subregion IV (Miralles *et al.* 2014) (Figs 1A–B). The grain samples were reduced from 1 kg to 250 g with a grain divider and later reduced to 200 grains for *Fusarium* species isolation. At the sampling point located at 36°30′54.36′′S, 62°8′35.88′′W, we found that approximately 15% of rye spikes had characteristic FHB symptoms (bleaching glumes that sometimes became necrotic and some grains had pale brown discoloration and necrotic areas with mycelial growth) (Fig. 1C). The symptomatic grains were surface sterilized (70% EtOH for 2 min; 5% NaClO for 2 min), rinsed twice in sterilized distilled water, plated on 2% potato dextrose agar (PDA) and incubated at 24°C (±2°C) in a 12 h dark/light cycle. Three fungal colonies morphologically similar to *Fusarium* were observed after 5 days of incubation. For identification, all isolates were taken and purified through monosporic culture and transferred onto PDA and carnation leaf agar (CLA) to grow under the same conditions as described above. Identification of species, based on morphological traits was carried out for all the isolates according to Leslie and Summerell (2006). A pathogenicity test was conducted to check Koch's postulates. A hand sprayer was used to inoculate spikes in potted plants (one plant per pot with three replications) during flowering stage with 5 ml spore suspension (1×10^5 conidia · ml^{-1}). Control pots were sprayed with sterile distilled water at random in different potted rye plants. All of the plants were entirely covered with polyethylene bags and incubated for 48 h in a greenhouse with ≥80% humidity and at 25°C (±2°C). The visual evaluation of the disease was performed 21 days after inoculation, observing the characteristic FHB symptoms in the spikelets.

Genomic DNA from the monosporic fungal culture (derived from one conidium) morphologically identified as *F. graminearum* was extracted using the CTAB method described by Stenglein and Balatti (2006). Portions of the reductase (RED) and trichothecene 3-O-acetyltransferase (*tri101*) genes were amplified by PCR and then one representative isolate was sequenced to identify the isolates to species level according to O´Donnell *et al.* (2000). PCR reactions were carried out using 10–20 ng of genomic DNA in a total volume of 25 µl containing 10X reaction buffer, 0.5 µm of the respective primers, 200 µm of each dNTP (Inbio-Highway, Tandil, Argentina), 2.5 mm $MgCl_2$ and 1.25 U of *Taq* DNA polymerase (Inbio-Highway, Tandil, Argentina). PCR conditions were performed in a XP Thermal cycler (Bioer Technology Co.) with the following conditions: 95°C for 2 min, followed by 30 cycles at 94°C for 30 s, 30 s at 57°C (for RED) and 62°C (for tri101), 72°C for 5 min and finally an elongation step at 72°C for 10 min. PCR products were purified by PureLink PCR purification kit (Invitrogen, Leohne, Germany) and DNA sequencing, from the paired end of the fragments was carried out using Big Dye Termination version 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA) in an Applied Biosystems Sequencer (ABI/Hitachi Genetic Analyzer

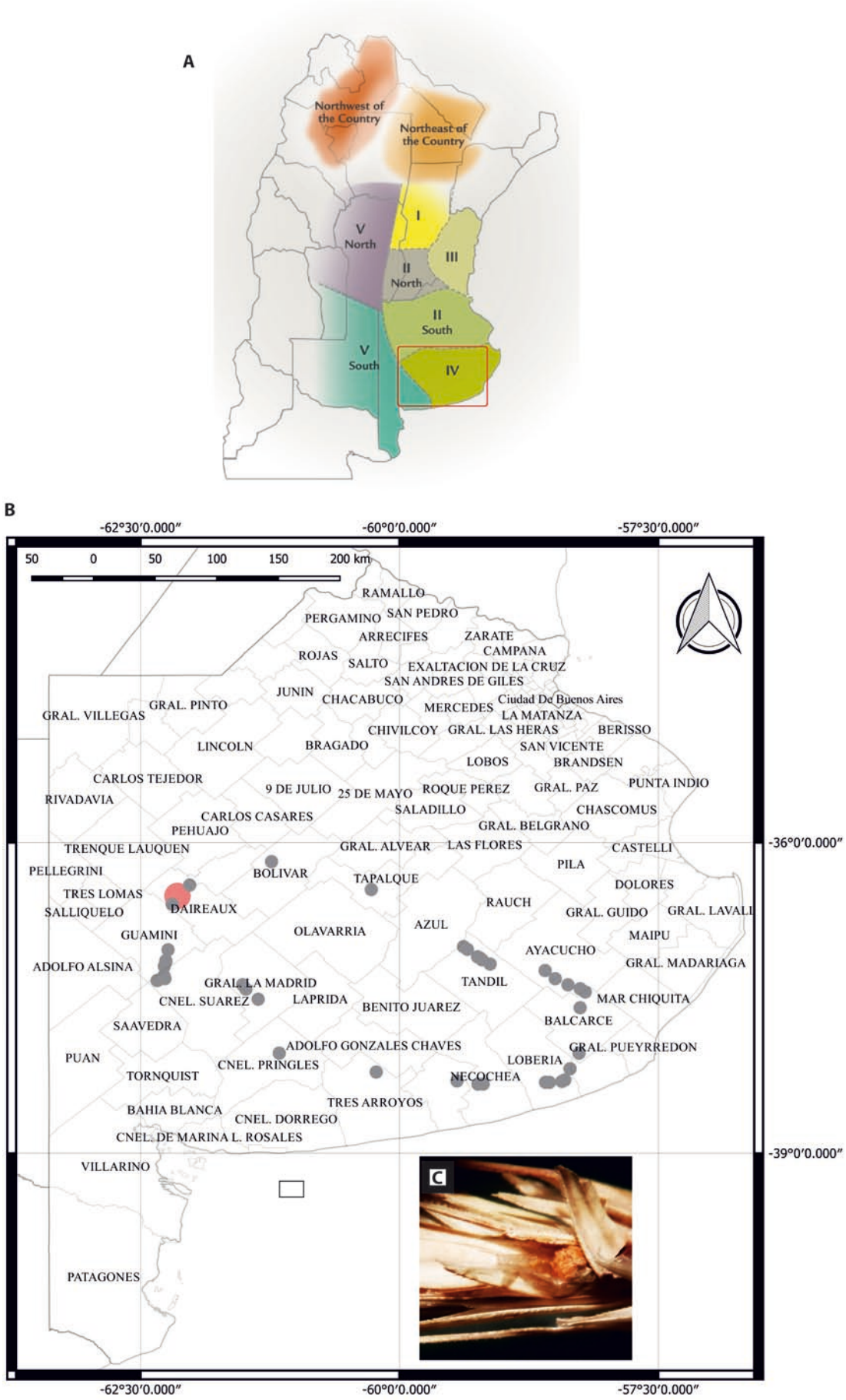

Fig. 1. A – map of Argentina's wheat growing regions taken from Trigo Argentino (2018). The square shows subregion IV where the samples were taken; B – sampling map of different locations in Buenos Aires province, Argentina, during the 2017/2018 harvest season. The pink circle shows the place where the isolation of *Fusarium graminearum* s.s. was found (Daireaux – 36°30′54′′S; 62°8′35′′W, Buenos Aires, Argentina); C – typical FHB symptoms on rye spike

3130). The final sequences were compared with those previously published in the NCBI website by using the BLASTN tool and finally submitted to GenBank.

In addition, the genetic potential of the *F. graminearum* s.s. isolates to produce toxins was determined. For this purpose, NIV and DON genotypes were differentiated by targeting different genes. First, primers based on the *tri13* gene were used: Tri13NIVF and Tri13NIVR for NIV genotypes and Tri13DONF and Tri13DONR for DON genotypes with the cycler conditions described by Chandler *et al.* (2003). Secondly, the NIV, DON and the two acetylates (3-ADON and 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 (Tri7F340, Tri7R965, 3551H, 4056H, Tri3F971, Tri3R1679, Tri3F325, Tri3R1679). The PCR conditions were performed as described by Quarta *et al.* (2006). In addition, the potential of the isolates to produce ZEA was determined using PKS4 primers targeting the PKS gene according to Meng *et al.* (2010). Two *F. graminearum* s.s. (15/15 and B26.1) previously characterized by Castañares *et al.* (2016) as 15-ADON and ZEA genotype, one 3-ADON *F. austramericanum* (NRRL2903), one NIV *F. meridionale* isolate (NRRL 28436) and one *F. poae* isolate (Hsu1a) were used as controls. All PCR reactions were performed twice. Products were examined by electrophoresis in 1.5% (w/v) agarose gels containing GelRedTM (Biotium, Hayward, CA, USA) at 80 V in 19 Trisborate–EDTA buffer for 2 h 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).

Fusarium graminearum isolates grow rapidly and produce large amounts of mycelia (white to pale orange) on PDA at 5 days under the conditions described above. On CLA, sporodochia are sparsed with macroconidia which are relatively slender with 5–6 septate. Clamydospores were observed and they were frequently visualized in the macroconidia. Microconidia were not observed. Based on morphological characteristics, the fungus was identified as *F. graminearum* (Schw.). Afterwards, the RED (accession N° MH753696) and tri101 (accession N° MH753699) sequences showed 99% similarity with the *F. graminearum* s.s. sequences available in the NCBI database confirming the previous morphological identification. Koch´s postulates were performed to confirm that the symptoms on the spikelet were attributed to the presence of *F. graminearum* s.s. After spray inoculation, spikelets were photographed at: 2 days post-inoculation (dpi), 7 dpi, 14 dpi and 21 dpi, to observe the fungus development. At 21 dpi, control spikelets were asymptomatic, while inoculated spikelets showed anther necrosis at 7 dpi and a large amount of mycelia at 21 dpi (Fig. 2). The fungus on symptomatic kernels was re-isolated and identified morphologically as *F. graminearum*.

Fusarium graminearum species complex (FGSC) is composed of at least 16 phylogenetically different species. *Fusarium graminearum* s.s. is the most common and widespread member of the FGSC, prevalent in America and Europe, but also reported worldwide. Other FGCS members are geographically more restricted to some countries of the world. In Argentina, FHB in wheat and barley seems to be caused exclusively by *F. graminearum* s.s., while the presence of other FGSC members has not been reported yet (Reynoso *et al.* 2011; Castañares *et al.* 2016). Among FGSC members, some species are able to produce different mycotoxins according to the presence of specific *Fusarium* species. There are no genotype production profiles for *F. graminearum* s.s. of isolates from rye in Argentina. Studies on *Fusarium* populations from different small cereal crops in the wheat cropping area in Argentina, reported that *F. graminearum* s.s. isolates were predominantly 15-ADON producers (Fernández Pinto *et al.* 2008; Alvarez *et al.* 2009; Reynoso *et al.*

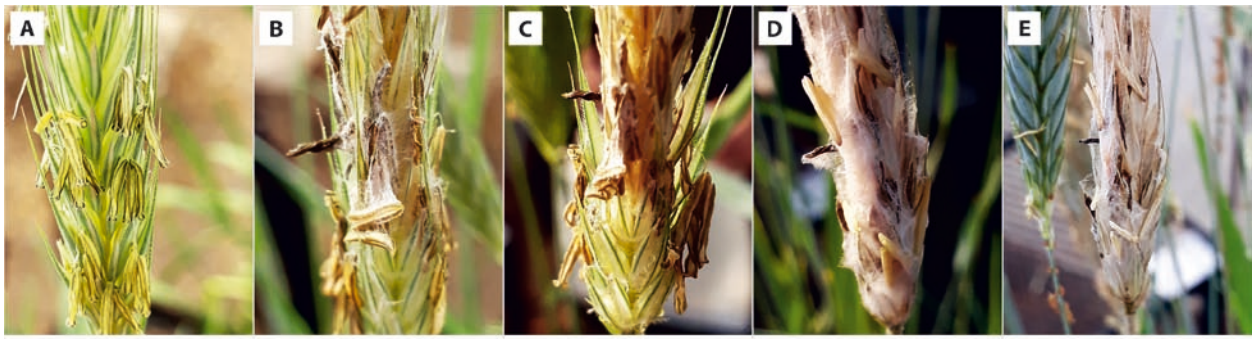

Fig. 2. *Fusarium* head blight progress in spikes inoculated with *Fusarium graminearum* s.s. isolates collected on rye under field conditions during the 2017 growing season in Argentina. A – anthesis, B – 2 days post-inoculation, C – 7 days post-inoculation, D – 14 days post-inoculation, E – 21 days post-inoculation

2011; Castañares *et al.* 2013). Our results showed that *F. graminearum* s.s. isolated from rye grains has the genetic potential to produce DON based on the amplification of 282 bp and 525 bp for primer targeting *tri13* and *tri3* genes related to DON production, while no amplification was observed for NIV primers. In addition, a fragment of 280 bp associated with the potential to produce ZEA was observed (Fig. 3).

To our knowledge, based on very scarce literature concerning FHB on rye (Brumana 1938; Carranza 1971), this is the first report of *F. graminearum* s.s associated with rye kernels in Argentina. Considering world production levels and the fact that rye is a minor cereal, these results are important due to increased global concern about food security and mycotoxin contamination, mainly by the *Fusarium* genus. Global climate change and conservation tillage practices could increase the impact of this disease in future scenarios, therefore constant monitoring is necessary to prevent mycotoxin contamination with its potential impact on human and animal health.

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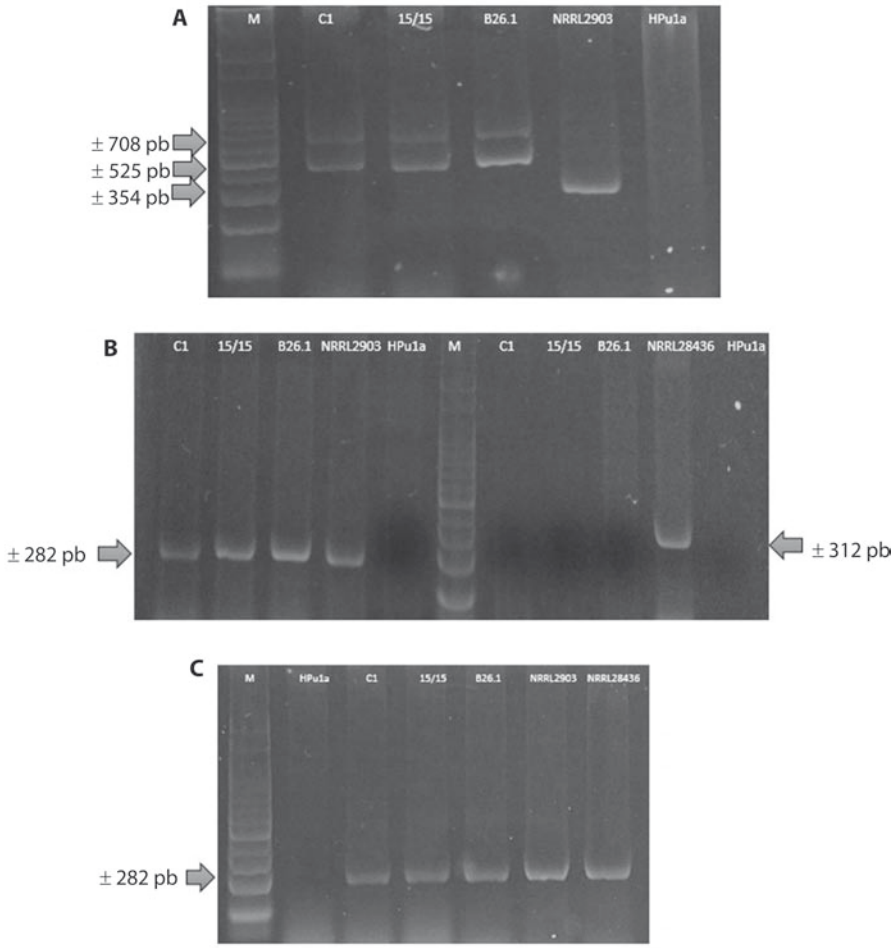

Fig. 3. Potential production of mycotoxins by *Fusarium graminearum* s.s. A – amplification of the deoxynivalenol (DON), 3-ADON, 15-ADON and nivalenol (NIV) specific fragments by multiplex PCR, B – amplification of the DON (left) and NIV (right) specific fragments by using *tri13* primers set, C – amplification of the zearalenone (ZEA) specific fragment.

M – molecular 100-bp marker, C1 – *F. graminearum* s.s. from this study, 15/15 and B26.1 – *F graminearum* s.s. (15-ADON/ZEA controls), NRRL2903 – *F. austroamericanum* (3-ADON/ZEA controls), NRRL28436 – *F. meridionale* (NIV/ZEA controls), HPu1a – *F. poae* (negative control). NRRL isolates were kindly provided by the ARS Culture Collection

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