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Development of a PCR-RFLP method based on the transcription elongation factor 1-α gene to differentiate *Fusarium graminearum* from other species within the *Fusarium graminearum* species complex

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ABSTRACT

Fusarium head blight (FHB) is a destructive disease of cereals crops worldwide and a major food safety concern due to grain contamination with trichothecenes and other mycotoxins. *Fusarium graminearum*, a member of the *Fusarium graminearum* species complex (FGSC) is the dominant FHB pathogen in many parts of the world. However, a number of other *Fusarium* species, including other members of the FGSC, may also be present for example in Argentina, New Zealand, Ethiopia, Nepal, Unites States in cereals such as wheat and barley. Proper species identification is critical to research aimed at improving disease and mycotoxin control programs. Identification of Fusarium species is are often unreliable by traditional, as many species are morphologically cryptic. DNA sequence-based methods offer a reliable means of species identification, but can be expensive when applied to the analyses of population samples. To facilitate identification of the major causative agent of FHB, this work describes an easy and inexpensive method to differentiate *F. graminearum* from the remaining species within the FGSC and from the other common *Fusarium* species causing FHB in cereals. The developed method is based on a PCR-RFLP of the transcription elongation factor (*TEF 1-α*) gene using the restriction enzyme *BsaH*I.

1. Introduction

Wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) are the most important winter crops in Uruguay. They constitute a very important economic activity in the country since both, wheat and barley are raw material for national industries, generating products with high added value. Grain yields in both cases can reach 3600 kg/ha (MGAP- OPYPA, 2016), but in certain years losses due to *Fusarium* Head Blight (FHB) constitute major constraints to production (Díaz and Pereyra, 2011). FHB is a destructive disease of cereals crops worldwide and a major food safety concern due to grain contamination with trichothecenes and other mycotoxins, of which deoxynivalenol (DON) is the most prevalent in Uruguay (Pan et al., 2013; Piñeiro and Silva, 1997).

Fusarium graminearum is the dominant FHB pathogen in many parts of the world. However, a number of *Fusarium* species, including other members of the *Fusarium graminearum* species complex (FGSC), may predominate in some regions or on some crops (Barreto et al., 2004; Bottalico and Perrone, 2002; Harrow et al., 2010; O'Donnell et al., 2004, 2008; Sarver et al., 2011; Starkey et al., 2007; Yli-Mattila et al., 2009). In Uruguay, Umpiérrez-Failache et al. (2013) found that *F. graminearum* was the main causative agent of FHB in wheat, however other species of FGSC (*Fusarium asiaticum*, *Fusarium brasilicum*, *Fusarium cortaderiae* and *Fusarium austroamericanum*) were also detected at low frequencies. *Fusarium graminearum* was also the main infective specie in Uruguayan barley, with *Fusarium poae, Fusarium avenaceum, Fusarium tricinctum* and *Fusarium equiseti* also present (Pereyra and Dill-Macky, 2010).

Species of FGSC typically produce type B trichothecenes of which the most commonly reported are deoxynivalenol (DON), nivalenol (NIV) and their derivatives, particularly 3-acetyl and 15-acetyl deoxynivalenol (3ADON and 15ADON) as well as 4-acetyl nivalenol (4ANIV). Lately, Varga et al. (2015) reported that some strains identified as *Fusarium graminearum sensu stricto* produced a

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new trichothecene named NX-2 and none of the previously reported type B trichothecenes. In this regard, FGSC isolates have been classified into 4 chemotypes (15ADON, 3ADON, NIV, and NX2) based on the profile of toxins produced in axenic cultures. Trichothecene chemotype differences are strain specific (Miller, 1991), but have been maintained by balancing selection such that many species display shared trichothecene chemotype polymorphism (Ward et al., 2002). Nevertheless, trichothecene chemotype diversity is structured biogeographically and correlated with species differences (Vaughan et al., 2016).

Proper species identification is critical to research aimed at improving disease and mycotoxin control programs. Traditional phenotypic methods are often unreliable for species identification in *Fusarium*, as many species are morphologically cryptic. DNA sequence-based methods offer a reliable means of species identification, but can be expensive when applied to the analyses of population samples (O'Donnell et al., 2015). PCR based approaches developed from the growing databases of DNA sequence variation offer an inexpensive and high-throughput option to generate reliable species identifications. However, these must be based on proper species circumscriptions and adequate sampling of diversity. For example, primers for the detection of *F. culmorum* or *F. graminearum sensu lato* were designed and validated by Nicholson et al. (1998). However, the primers they developed for specific detection of *F. graminearum* (Fg16F and Fg16R) were designed prior to the recognition and description of this morphospecies as a complex. To date, FGSC comprises16 phylogenetically distinct species including (O'Donnell et al., 2008; Sarver et al., 2011) As such, the capacity to amplify all species within the FGSC was not demonstrated, and the use of these primers for specific detection of *F. graminearum sensu stricto* was not evaluated. To facilitate identification of the major causative agents of FHB, this work describes an easy and inexpensive method to differentiate *F. graminearum* from the remaining species within the FGSC, based on a PCR-RFLP of the TEF 1-α gene.

2. Materials and methods

2.1. Fungal cultures

Strains representing the different species of the FGSC as well as *Fusarium roseum*, *Fusarium poae*, *Fusarium acuminatum* and *Fusarium sporotrichioides* were kindly provided by the Agricultural Research Service (NRRL) culture collection from the United State Department of Agriculture - Agricultural Research Service, (USDA-ARS) (Table 1). In addition, 151 *Fusarium* isolates obtained from Uruguayan wheat and identified to species by multilocus genotyping (MLGT) (Umpiérrez-Failache et al., 2013) were also included in this study. Those isolates were identified as *Fusarium graminearum* (130), *Fusarium asiaticum* (11), *Fusarium austroamericanum* (1), *Fusarium brasilicum* (2) and *Fusarium cortaderiae* (7).

2.2. Genomic DNA extraction

All isolates were grown on PDA medium (Oxoid, England) and incubated for seven days at 25 °C. Mycelium was recovered with a sterile spatula and total DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ (ZymoResearch, USA). The quality of DNA was evaluated by agarose gel electrophoresis (Sambrook et al., 1989). Double stranded DNA was quantified using QubitdsDNA HS Assay Kit (Invitrogen, USA) in a QubitFluorometer (Invitrogen, USA).

Table 1
NRRL culture collection strains.

Species	Reference number
Fusarium austroamericanum	NRRL 2903
Fusarium cortaderiae	NRRL 29297
Fusarium asiaticum	NRRL13818
Fusarium boothii	NRRL 26916
Fusarium brasilicum	NRRL 31281
Fusarium meridionale	NRRL 28436
Fusarium gerlachii	NRRL 36905
Fusarium louisianense	NRRL 54196
Fusarium ussurianum	NRRL 45665
Fusarium vorosii	NRRL 37605
Fusarium nepalense	NRRL 54220
Fusarium acaciae-mearnsii	NRRL 26752
Fusarium mesoamericanum	NRRL 25797
Fusarium aethiopicum	NRRL 46710
Fusarium sp.	NRRL 34461
Fusarium graminearum	NRRL 31084
Fusarium roseum	NRRL 6470
Fusarium poae	NRRL 13714
Fusarium sporotrichioides	NRRL 36147
Fusarium acuminatum	NRRL 3299

2.3. Use of Fg16 primers to detect species from the FGSC

DNA samples from FGSC isolates provided by the NRRL collection were used as target in PCR assays with primers Fg16F and Fg16R designed by Nicholson et al. (1998) in order to evaluate the amplification of all species currently included in the complex. *Fusarium roseum* NRRL 6470, *Fusarium poae* NRRL 13714, *Fusarium acuminatum* NRRL 36147 and *Fusarium sporotrichioides* NRRL 3299 were included in the assay as non-target species. Amplification was performed in 25 μL of reaction mixture containing 1X Buffer (Fermentas International Inc., Canada), 0.5 μM of each primer, 0.2 mM of dNTP, 0.5 U of Taq polymerase (Fermentas International Inc., Canada), and 50 ng of DNA. PCR was performed in a MultiGene™ thermocycler (Labnet International Inc., Edison, NJ, USA) under the following conditions: initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were separated on 0.8% agarose gels containing ethidium bromide (5 μg/mL) and the resulting DNA bands were visualized under UV light. The various sizes of the obtained amplicons were estimated by comparison with a DNA standard marker (GeneRuler ™1Kb DNA ladder, Fermentas International Inc., Canada).

2.4. Development of a method based on PCR-RFLP of TEF1-α gene to differentiate Fusarium graminearum from other species within the FGSC

TEF 1-α sequences corresponding to strains in Table 1 representing the FGSC were retrieved from GenBank or Fusarium ID database (http://isolate.fusariumdb.org/blast.php) (Geiser et al., 2004; Park et al., 2011). The obtained sequences were aligned using Mega version 6 (Tamura et al., 2013) and trimmed in order to obtain fragments of the same size. The fragments were analysed using Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/) and Nebcutter 2.0 (http://tools.neb.com/NEBcutter2/) to identify a restriction enzyme which could produce a different RFLP pattern for *Fusarium graminearum* in comparison with all other species belonging to the FGSC.

In-silico RFLP results were confirmed empirically by analyzing the isolates listed in Table 1 representing the species of FGSC. Amplification of the TEF 1-α gene was carried in 25 μL of reaction