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Species composition, toxigenic potential and aggressiveness of Fusarium isolates causing Head Blight of barley in Uruguay

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ABSTRACT

Fusarium Head Blight (FHB) is a major constraint to barley production that substantially reduces yield and grain quality. FHB is also a major food safety concern because FHB pathogens contaminate grain with trichothecenes and other mycotoxins. DNA sequence-based analyses and *in-vitro* toxin assessments were used to characterize the species and trichothecene chemotype composition of FHB pathogens on barley in Uruguay. *F. graminearum* was the dominant species (89.7%), and three other members of the *F. graminearum* species complex (FGSC) were identified as FHB pathogens of barley in Uruguay for the first time. Other minor contributors to FHB species diversity included *F. poae, F. avenaceum*, *F. pseudograminearum* and an unnamed species from the *F. incarnatum-equiseti* species complex (FIESC). Most isolates (89.7%) had the 15-acetyldeoxynivalenol (15-ADON) trichothecene type. However, the results expanded the known area of occurrence within Uruguay for the nivalenol (NIV) toxin type, which was observed among isolates from three species of the FGSC, *F. pseudograminearum*, and *F. poae*. Isolates with the 3-acetyldeoxynivalenol (3-ADON) or NX-2 toxin types were not observed, although a previously published multilocus genotyping assay was updated to identify NX-2 strains. Analyses of population structure and comparisons with FHB isolates from wheat in Uruguay indicated that *F. graminearum* constitutes a single genetic population with no evidence of population differentiation related to the sampled hosts. Inter and intraspecific differences were observed in aggressiveness toward four barley genotypes with different levels of resistance to FHB, and in general nivalenol producers were the least aggressive isolates. Sensitivity to metconazole was approximately 10 times higher than was detected for tebuconazole. This is the first report regarding tebuconazole and metconazole sensitivity for *Fusarium* species causing FHB in barley in Uruguay, and constitutes an important starting point for monitoring temporal or spatial changes in FGSC sensitivity, which is critical to define FHB management practices.

1. Introduction

Barley (*Hordeumvulgare* L.) represents the second most important winter crop in Uruguay. In the last ten years, an average of 112,000 ha were planted with barley annually, and grain yields varied from 1878 to 3510 kg/ha (OPYPA, 2015). In Uruguay, barley production occurs in the southwestern portion of the country, and is particularly concentrated in the districts of Colonia and Soriano. Yield and quality losses due to Fusarium Head Blight (FHB) are major constraints to barley production (Díaz M., 2011) In addition, FHB is a major food safety concern because FHB pathogens can contaminate grain with trichothecenes and other mycotoxins, of which deoxynivalenol is the most prevalent in Uruguay (Pan et al., 2013; Piñeiro, 1997).

Many species of *Fusarium*, including those in the *F. sambucinum*, *F.tricinctum*, and *F. incarnatum-equiseti* species complexes (FSAMSC, FTSC, and FIESC) (O'Donnell et al., 2013) are able to incite FHB symptoms. However, the sixteen described species within the *Fusarium graminearum* species complex (FGSC), which is nested within the broader FSAMSC, are the principal causal agents of FHB in small grain cereals, including barley (Castañares et al., 2014; O'Donnell et al., 2004, 2008; Sarver et al., 2011; Starkey et al., 2007; Yli-Mattila et al., 2009). Members of the FGSC typically produce one of three strain-

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specific profiles (chemotypes) of type B trichothecene mycotoxins. These include the nivalenol (NIV), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) chemotypes (Miller et al., 1991). Recently, a small number of *F. graminearum* isolates have been found to produce a novel type A trichothecene, NX-2, due to genetic variation in the trichothecene biosynthetic gene *TRI1* (Liang et al., 2014; Varga et al., 2015).

FGSC species and trichothecene chemotype diversity is biogeographically structured and can vary significantly among different hosts in a given region (Lee et al., 2009; Boutigny et al., 2011; Gomes et al., 2015; Beccari et al., 2016). Knowledge of species and chemotype diversity in relation to aggressiveness and fungicide sensitivity among FHB pathogens in a particular region is important for the assessment of the trichothecene mycotoxins that could be present in grain and for the deployment of effective management strategies. In a previous study (Umpiérrez et al., 2013), found that *F. graminearum* with the 15ADON trichothecene type was the predominant contaminant in wheat grains. However, no such information is available for barley isolates although wheat and barley share similar production zones in southwestern Uruguay. The aims of this study were i) to determine the species and trichothecene chemotype composition of FHB pathogens of barley in traditional production areas in Uruguay and ii) to assess variation among isolates in terms of aggressiveness on barley and sensitivity to the triazole fungicides metconazole and tebuconazole.

2. Materials and methods

2.1. Barley samples

One hundred and fifty four grain samples (0.2 kg) of barley were collected from different regions in southwestern Uruguay after harvest during 2012, in which FHB was at epidemic levels. Samples were provided by Malteria Uruguay-AMBEV, Malteria Oriental S. A. and Copagran, from Colonia (95), Soriano (41), Río Negro (8), Flores (6) and San José (4).

2.2. Fungal isolates

One hundred kernels of each sample were surface disinfected in a 0.5% aqueous solution of sodium hypochlorite for 5 min, rinsed twice in sterile distilled water and dried on sterile filter paper. Twenty kernels per plate were grown on pentachloronitrobenzene (PCNB) agar medium (Nash and Snyder, 1962) supplemented with 34 μg/ml of chloramphenicol (Sambrook et al., 1989). Plates were incubated at 25 °C in darkness for four to six days. Monosporic cultures were obtained on Potato Dextrose Agar (PDA, Oxoid, England) from colonies identified macro- and microscopically as *Fusarium* spp. (Leslie and Summerell, 2008). No more than two isolates were collected from each sample. Pure cultures were subsequently stored on PDA slants at 4 °C.

2.3. Genomic DNA extraction

All isolates were grown on PDA medium (Oxoid, England) and incubated during 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). DNA quality was assessed by agarose gel electrophoresis (Sambrook et al., 1989). Double stranded DNA was quantified using a Qubit dsDNA HS Assay Kit (Invitrogen, USA) in a Qubit Fluorometer (Invitrogen, USA).

2.4. Species and trichothecene chemotype determination

Species identification and assessment of trichothecene type were performed by multilocus genotyping (MLGT) using a Luminex 100 flow cytometer as previously described (Sarver et al., 2011; Ward et al., 2008). The previously published MLGT assay based on 48 probes was updated to identify strains with the novel NX-2 trichothecenechemotype by inclusion of primers for amplification of a portion of the-*TRI1*gene (LxTri1F 5′-GCTCTCATCACCAGTTTGCAG- 3′ and LxTri1R 5′-CGTGTTGAGATTCTAGTGACC-3′)and novel probes for the identification of genetic variation within *TRI1* that is specific to isolates with the NX-2 chemotype (M150-Tri1A-NX2 5′-CTTTCTCATACTTTCAACT AATTTTATCCGTCGTACGAAAGAAGCTGA-3′ and M153-Tri1D-NX2 5′-TACATTCAACACTCTTAAATCAAACACTCTGGCCGATGAAATCAA GAA-3′). The assay also included a positive control probe to detect the *TRI1*amplicon (5′-CTTAACATTTAACTTCTATAACACATGCTCGTGCAG TCTCAGAAGTCC-3′). Isolates that could not be identified by MLGT were analyzed by partial sequencing of the Transcription Elongation Factor 1 alpha (TEF-1α) gene (O'Donnell et al., 1998). Sequence similarity searches were performed with the BLAST network service of the Fusarium ID database (http://isolate.fusariumdb.org/blast.php) (Geiser et al., 2004; Park et al., 2010) and GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Population structure and diversity of F. graminearum in Uruguay

Genetic variation at eight variable number tandem repeat (VNTR) loci (Aamot et al., 2015; Suga et al., 2008) was determined as described by Bec et al. (2015) for *F. graminearum* isolates recovered from barley, and for a subset of *F. graminearum* isolates from a previous study of wheat in Uruguay (Umpiérrez et al., 2013). Isolates that failed to produce amplicons for one or more loci were excluded from analyses. GenAlEx version 6.5 (Peakall and Smouse, 2012) was used to test for population structure in relation to host (barley versus wheat) and geographic origin (districts represented by at least 30 F. *graminearum* isolates) within Uruguay. The extent of genetic differentiation between groups of isolates was estimated by calculating φ_{PT} (a standardized equivalent of F_{ST} for haploid data), with significance assessed using 10,000 random permutations of the data. GenAlEx was also used to calculate unbiased gene diversity(*H*, measured within populations and standardized by sample size) and the number of effective alleles across loci (*Ne*) for each subgroup defined by host or geographic origin.

2.6. Nivalenol production

Quantification of nivalenol production was assessed as described by Castañares et al. (2014) with modifications, for all *F. poae* isolates and for isolates from the FGSC with the NIV genotype as determined by MLGT. Twenty-five grams of rice were placed in 250 ml Erlenmeyer flasks, moistened with 15 ml of distilled water and then autoclaved for 15 min at 121 °C. After cooling, each flask was inoculated with a 3-mm diameter agar disc taken from the margin of a colony of each isolate grown on PDA at 25 °C for three days. For each isolate, the experiment was repeated twice and the average nivalenol production and confidence intervals were calculated using Microsoft Excel. Negative controls 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. After incubation each culture was placed in a sterile plastic bag, and weighed. A volume of water representing four times the weight of each sample was added to each bag and the mixtures were homogenized for 3 min in a stomacher. The homogenates were filtered through paper and two mililiters of the filtered extract was applied to DON-NIV™ WB immunoaffinity columns (Vicam, Waters Corporation) in order to isolate the mycotoxins. The obtained solutions were analyzed by HPLC UV, as described by Visconti and Bottalico (1983) to quantify nivalenol levels produced by each isolate.

2.7. Aggressiveness tests

The aggressiveness of 15 isolates representative of species isolated in this survey was assessed by spray inoculation on four barley