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Regional differences in species composition and toxigenic potential among Fusarium head blight isolates from Uruguay indicate a risk of nivalenol contamination in new wheat production areas

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ABSTRACT

Members of the *Fusarium graminearum* species complex (FGSC) are the primary cause of Fusarium head blight (FHB) of wheat, and frequently contaminate grain with trichothecene mycotoxins that pose a serious threat to food safety and animal health. The species identity and trichothecene toxin potential of 151 FGSC isolates collected from wheat in Uruguay were determined via multilocus genotyping. Although *F. graminearum* with the 15ADON trichothecene type accounted for 86% of the isolates examined, five different FGSC species and all three trichothecene types were identified in this collection. This is the first report of *Fusarium asiaticum*, *Fusarium brasilicum*, *Fusarium cortaderiae*, and *Fusarium austroamericanum* from Uruguay. In addition, we observed significant ($P < 0.001$) regional differences in the composition of FGSC species and trichothecene types within Uruguay. Isolates of *F. graminearum* with the 15ADON type were the most prevalent in western provinces (95%), while *F. asiaticum* (43%) and the NIV type (61%) predominated in the new wheat production zone in Cerro Largo along Uruguay's eastern border with Brazil. *F. graminearum* isolates (15ADON type) were significantly ($P < 0.005$) more aggressive on wheat than were isolates from the other species examined (NIV or 3ADON types). However, *F. graminearum* isolates (15ADON type) were significantly ($P < 0.05$) more sensitive to tebuconazole than isolates from other species (NIV type). These results document substantial heterogeneity among the pathogens responsible for FHB in Uruguay. In addition, the regional predominance of the NIV trichothecene type is of significant concern to food safety and indicates that additional monitoring of nivalenol levels in grain may be required.

1. Introduction

Fusarium head blight (FHB) is a destructive disease of wheat worldwide, and is primarily attributable to members of the *Fusarium graminearum* species complex (FGSC), which encompasses at least sixteen recognized species (O'Donnell et al., 2008, 2004; Sarver et al., 2011; Starkey et al., 2007; Yli-Mattila et al., 2009). In Uruguay, FHB represents one of the main constraints for wheat production, and moderate to severe outbreaks have occurred in one of every two years over the past decade (Díaz de Ackermann and Kohli, 1997; Perea and Díaz, 1980; Pereyra, 2003). A particularly severe outbreak in 2002 caused substantial losses in grain yield and reductions in quality attributable to the presence of mycotoxins in harvested grain (Pereyra et al., 2006). In the last five years, the highest levels of FHB in wheat were during 2009 and 2013 (Grupo Trigo, 2013).

Members of the FGSC can produce various mycotoxins; however, trichothecenes act as virulence factors on wheat (Jansen et al., 2005; Proctor et al., 1995) and are frequently found in grain (Miller, 2008). In addition, trichothecenes are a significant concern for human and animal health because they are cytotoxic, inhibit protein synthesis, and can modulate immune function (Pestka, 2010). Three trichothecene chemotypes have been recognized among members of the FGSC based on the profile of trichothecene metabolites produced by individual strains. The NIV chemotype includes nivalenol and 4-acetylnivalenol producers, whereas the 3-ADON and 15-ADON chemotypes include isolates that produce deoxynivalenol and primarily 3-acetyldeoxynivalenol or 15-acetyldeoxynivalenol respectively (Miller and Greenhalgh, 1991). These three chemotypes have been maintained through multiple speciation events by balancing selection, indicating that chemotype differences are adaptive (Ward et al., 2002).

Potential differences in host preference have been reported for members of the FGSC (Boutigny et al., 2011; Lee et al., 2009; Nielsen et al., 2011, 2012; Sampietro et al., 2011) and substantial geographic variation in FGSC species and trichothecene chemotype diversity have

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been observed (Miller, 2002; Monds et al., 2005; Nielsen et al., 2012; O'Donnell et al., 2000; Suga et al., 2008; Ward et al., 2008; Yli-Mattila et al., 2009). Differences in toxicity between NIV and DON have also been reported (Minervini et al., 2004) and the Scientific Committee on Food of the European Union (SCF) recommended a tolerable daily intake (TDI) of 1 μg/kg body weight for DON and a provisional TDI of 0.7 μg/kg body weight for nivalenol (EU, 2006). In Uruguay, deoxynivalenol, but not nivalenol, levels have been regulated in wheat grains and in flour since 2001 (MSP, 2001). Therefore, knowledge of FGSC species and chemotype diversity in a particular region is important for the development of disease management strategies and assessment of the trichothecene mycotoxins that could be present in grain. However, information on the trichothecenes associated with wheat in Uruguay is very limited (Piñeiro et al., 1996), and there is essentially no data regarding the prevalence of different FGSC species in Uruguay. In addition, the major wheat growing area in Uruguay has recently expanded from the western border of the country, next to Argentina, into the center and the east of the country, near Brazil (Ernst, 2011) The objective of this study was to provide the information needed to improve disease management and toxin monitoring strategies in Uruguay by assessing the diversity of FGSC pathogens and trichothecene types associated with FHB of wheat in traditional and new wheat production areas in Uruguay, and by evaluating the aggressiveness and tebuconazole sensitivity of these FHB isolates.

2. Materials and methods

2.1. Wheat samples

Sixty-six grain samples (0.2 kg) of wheat were collected from different regions in Uruguay in four seasons (2002, 2009, 2011 and 2012). In 2002, samples were provided by the Ministerio de Ganadería Agricultura y Pesca (MGAP) from a national survey to detect DON contamination levels (62 isolates). In 2009, samples were provided by the Instituto de Investigación Agropecuaria (INIA) and individual farmers of the agricultural cooperative COPAGRAN (48 isolates). In 2011 and 2012, samples were provided by local farmers of Cerro Largo (17 isolates) and Lavalleja (11 isolates) and by the Facultad de Agronomia-Udelar in Montevideo (13 isolates).

2.2. Fungal isolates

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 Potato Dextrose Agar (PDA) supplemented with 34 μg/ml of chloramphenicol (Sambrook et al., 1989) and incubated at 25 °C in darkness for 4–6 days. Monosporic cultures on PDA, were isolated from colonies identified macro and microscopically as *Fusarium* spp. (Leslie and Summerell, 2006). No more than two isolates were collected from each sample. Pure cultures were subsequently stored on PDA slants at 4 °C. Representative isolates were accessioned into the U.S. Department of Agriculture, Agriculture Research Service (ARS) Culture Collection, Peoria, IL and assigned NRRL numbers. Histories for the 151 FGSC isolates included in this study are provided (Supplemental Table 1).

2.3. Genomic DNA extraction

All isolates were grown in 20 ml flasks containing 5 ml yeast extract sucrose (YES, 2% yeast extract, 15% sucrose) and incubated for 5 days at 25 °C prior to genomic DNA extraction. Genomic DNA extraction was performed according to Voigt et al. (1999) with minor modifications. The cultured mycelium was dried using a Buchner funnel or sterile filter paper and approximately 50% of the mycelium was collected and broken up by pipetting using 600 μl of CTAB lysis buffer. The mycelium/CTAB mixture was incubated at 65 °C for 60 min and pipetted up/down about 20 times before addition of 600 μl of chloroform. After centrifugation at maximum speed for 15 min, the aqueous phase was removed and placed in a clean tube; 400 μl of isopropyl alcohol was added and incubated for 20 min at 20 °C, centrifuged at maximum speed for 10 min, washed with 70% ethanol, dried and suspended in 100 μl sterile milli-Q DNAse free water. DNA quantitation was performed using Quant-iT ds DNA BR Kit (Invitrogen) and a Qubit Fluorometer (Invitrogen).

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 (Ward et al., 2008). MLGT was based on allele-specific primer extension (ASPE) using a 45 probe set described previously by Yli-Mattila et al. (2009), targeting species and trichothecene chemotype-specific genetic variation in six genes (Ward et al., 2008; Yli-Mattila et al., 2009). The statistical significance of temporal or regional differences in the frequencies of FGSC species and trichothecene types was assessed using Fisher's exact test.

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 BLAST network service of the Fusarium ID database (http://isolate.fusariumdb.org/blast.php) (Geiser et al., 2004; Park et al., 2011).

2.5. Aggressiveness tests

Aggressiveness of 11 isolates representative of all species and trichothecene types observed in the 2002 and 2009 surveys was assessed by single-floret inoculation on seven wheat genotypes with different levels of resistance to FHB. These included the susceptible cultivars Buck Guarani and INIA Mirlo, the moderately susceptible INIA Churrinche, the moderately susceptible to moderately resistant Catbird 1073, the moderately resistant INIA Tero and Sumai 3, and the resistant cultivar Frontana. Plants were grown in the greenhouse in plastic pots (150 by 150 mm, diam. by height) containing a 1:1:1 mixture of sand:soil:commercial substrate (Plantmax®, Eucatex, Sao Paulo, Brazil). Four to five seeds were planted per pot and thinned to two plants per pot when the seedlings were at the three leaf stage (Zadoks et al., 1974). Plants were maintained at 20–25 °C with alternating 12 h photoperiod. Plants were fertilized every week with soluble NPK (12-8-5, NPK + micronutrients, foliar fertilizer ISUSA, San José, Uruguay) applied at 30 ml per pot, using a solution of 400 ml fertilizer per liter of water. Inoculum from each *Fusarium* isolate was obtained from the single conidial cultures obtained previously. Conidia were produced on soybean medium (Pereyra and Dill-Macky, 2010) in Petri dishes for 12–14 days. Conidia were harvested by adding 10–15 ml of sterile deionized water to each plate and gently scraping the culture with a sterile glass rod. Inoculum concentration was adjusted to 5×10^4 conidia/ml. Inoculum viability was tested before inoculation. One to two spikes per pot (ten pots per isolate per wheat genotype) were inoculated at mid-anthesis using a micropipette dispensing 10 μl of the spore suspension in a spikelet positioned in the middle of each spike. Controls were mock-inoculated with sterile deionized water. Inoculated plants were incubated in a dew chamber at 20 to 22 °C with a 12-h photoperiod and 100% relative humidity for 72 h. After incubation, the plants were returned to the greenhouse and grown under the same light and temperature conditions used prior to inoculation. Disease severity was evaluated at 7, 14 and 21 days after inoculation (dpi) and expressed as the percentage of symptomatic spikelets per spike. Area under disease progress curve (AUDPC)

was calculated. The experimental layout was a randomized complete block design with 10 replicates (pot). Average percent severities were calculated for each replicate. Data were subjected to analysis of variance after testing assumptions of independence, normality and homoscedasticity using SAS (version 9.1, SAS Institute Inc., Cary, NC). Where the F ratio was significant ($P \leq 0.05$), differences among treatment means were separated using Tukey's test. Analyses were performed using Proc GLM of SAS (version 9.1, SAS Institute Inc., Cary, NC).

2.6. Sensitivity to tebuconazole

Sensitivity to tebuconazole was quantified by measuring the radial growth of the colonies grown on PDA (Becher et al., 2010; Tateishi et al., 2010) amended with different tebuconazole concentrations. The fungicide Folicur 25 was diluted in sterile water and incorporated into sterilized PDA to achieve concentrations of 0, 0.25, 1, 2 and 4 ppm. A mycelial plug (5 mm in diameter) of each isolate was taken from the periphery of a 3-day-old colony and placed on the center of a PDA plate amended with fungicide at each concentration. Two replicates for each concentration were used for each isolate. After 5 days of incubation at 25 °C in darkness, colony diameters were measured in two perpendicular directions. Effective inhibition of mycelial growth was determined for each fungicide concentration relative to control medium without fungicide. Effective concentration of tebuconazole leading to a 50% inhibition (EC_{50}) of mycelial growth was calculated on the basis of linear regression analysis of logit-transformed fungicide efficiency and the log10 transformed fungicide concentrations as described by Becher et al. (2010). The experiment was repeated twice and the average EC_{50} and confidence intervals were calculated using Microsoft Excel.

3. Results

3.1. Occurrence of FGSC species and trichothecene types

A total of 151 *Fusarium* isolates were characterized using the MLGT assay. Most of these isolates (86%, 130/151) were identified as *F. graminearum*, while we identified the remainder as *Fusarium asiaticum* (11), *Fusarium cortaderiae* (7), *Fusarium brasilicum* (2) and *Fusarium austroamericanum* (1). With the exception of a single 3ADON isolate from Soriano, all of the *F. graminearum* isolates had the 15ADON type. *F. cortaderiae*, *F. asiaticum* and *F. brasilicum* isolates shared the NIV type, while the single *F. austroamericanum* isolate (collected in Paysandú) had the 3ADON type. Three isolates identified morphologically as FGSC were found by MLGT and TEF-1α sequence to be species outside the FGSC (two *Fusarium acuminatum* and one *Fusarium lacertarum*). They were excluded from further analysis.

Significant differences ($P < 0.001$) in the composition of FGSC species and trichothecene types were observed between the major wheat production areas in western provinces and new wheat production areas in the eastern provinces of Cerro Largo and Lavalleja (Fig. 1). *F. graminearum* isolates with a 15ADON chemotype accounted for 95% of the isolates from western provinces, although a total of four *F. cortaderiae* (NIV chemotype) and a single *F. austroamericanum* (3ADON chemotype) were isolated from the western region of the country. In contrast, *F. graminearum* and the 15ADON type accounted for only 52% of the isolates from eastern provinces. This difference was most obvious in Cerro Largo, where *F. asiaticum* (43%) and the NIV chemotype (61%) were predominant. Collections in Cerro Largo were not available prior to 2009; however, no statistically significant differences in species or trichothecene composition were observed between populations collected from western provinces in 2002 and 2009 ($P = 0.3017$).

3.2. Aggressiveness

All isolates were capable of inducing FHB at 21 dpi; however variation in aggressiveness was observed among the FGSC isolates (range = 5.6 to 27.5%). Averaged over the seven wheat genotypes, *F. graminearum* isolates induced significantly ($P = 0.005$) higher FHB severity scores at 21 dpi than the rest of the species (Fig. 2). However, for one isolate of *F. asiaticum* (F. asiat. F-110) FHB severity scores were not significantly different from that obtained by *F. graminearum* isolate NRRL 36970. Although there were no significant isolate–wheat genotype interactions, there were significant differences ($P < 0.0001$) in AUDPC values and FHB severity scores at 7, 14 and 21 dpi among wheat genotypes. The highest values for all studied variables were observed on the susceptible cultivar Buck Guarani. Mean AUDPC values and FHB severity scores of the different isolates on resistant wheat genotypes were low and similar, indicating that these genotypes provided broad resistance to the FGSC species and trichothecene types examined. The lowest aggressiveness values were observed in resistant or moderately resistant cultivars Frontana, INIA Tero and Sumai3. However, mean AUDPC values observed for the resistant genotype Sumai3 were not significantly different from the moderately susceptible cultivar INIA Churrinche.

3.3. Sensitivity to tebuconazole

The EC50 values of tebuconazole for *F. graminearum* isolates with the 15ADON type (N = 42) were 0.07–0.98 μg/ml, with a mean of 0.29 μg/ml while the EC50 values for *F. asiaticum* and *F. cortaderiae* isolates with the NIV type (N = 14) were 0.24–1.73 μg/ml, with a mean of 0.61 μg/ml (Fig. 3). As a group, the *F. asiaticum* and *F. cortaderiae* isolates were significantly ($P < 0.05$) less sensitive to tebuconazole than were the *F. graminearum* isolates. However, when the *F. asiaticum* and *F. cortaderiae* isolates were considered separately, the differences in tebuconazole sensitivity were not statistically significant.

4. Discussion

This study revealed for the first time the *F. graminearum* species complex (FGSC) diversity responsible for FHB of wheat in Uruguay. *F. graminearum* appeared as the predominant species causing FHB in wheat. However, results also indicated that *F. asiaticum* and *F. cortaderiae* are important contributors to FHB infection and trichothecene contamination in new wheat production areas in the east of the country. Moreover, this is the first report of the presence of *F. cortaderiae*, *F. asiaticum*, *F. brasilicum* or *F. austroamericanum* in Uruguay.

Although *F. graminearum* is hypothesized to have originated in North America, this species has a global distribution (Sarver et al., 2011) and is known to be a common cause of FHB in South America (Ramirez et al., 2007; Scoz et al., 2009). *F. austroamericanum*, *F. brasilicum* and *F. cortaderiae*, were originally described from South American isolates and are hypothesized to have evolved in South America (Aoki et al., 2012). In contrast, *F. asiaticum* is endemic in Asia and is a common cause of FHB in China (Gale et al., 2002; Ji et al., 2007; Qu et al., 2007; Yang et al., 2008; Zhang et al., 2007), Japan (Karugia et al., 2009; Suga et al., 2008), Nepal (Sarver et al., 2011), Korea (Lee et al., 2009), and Russian Far East (Yli-Mattila et al., 2009) but this species has not previously been reported as a causative agent of FHB in South America.

Interestingly, all of the *F. asiaticum* isolates identified in this study were recovered from new wheat production areas that are near rice growing zones in eastern Uruguay. The authors of the first study documenting *F. asiaticum* as the cause of FHB in North American wheat hypothesized that this species may have been initially introduced from Asia on rice, subsequently moving onto wheat in regions of Louisiana where rice production is common (Gale et al., 2011). In

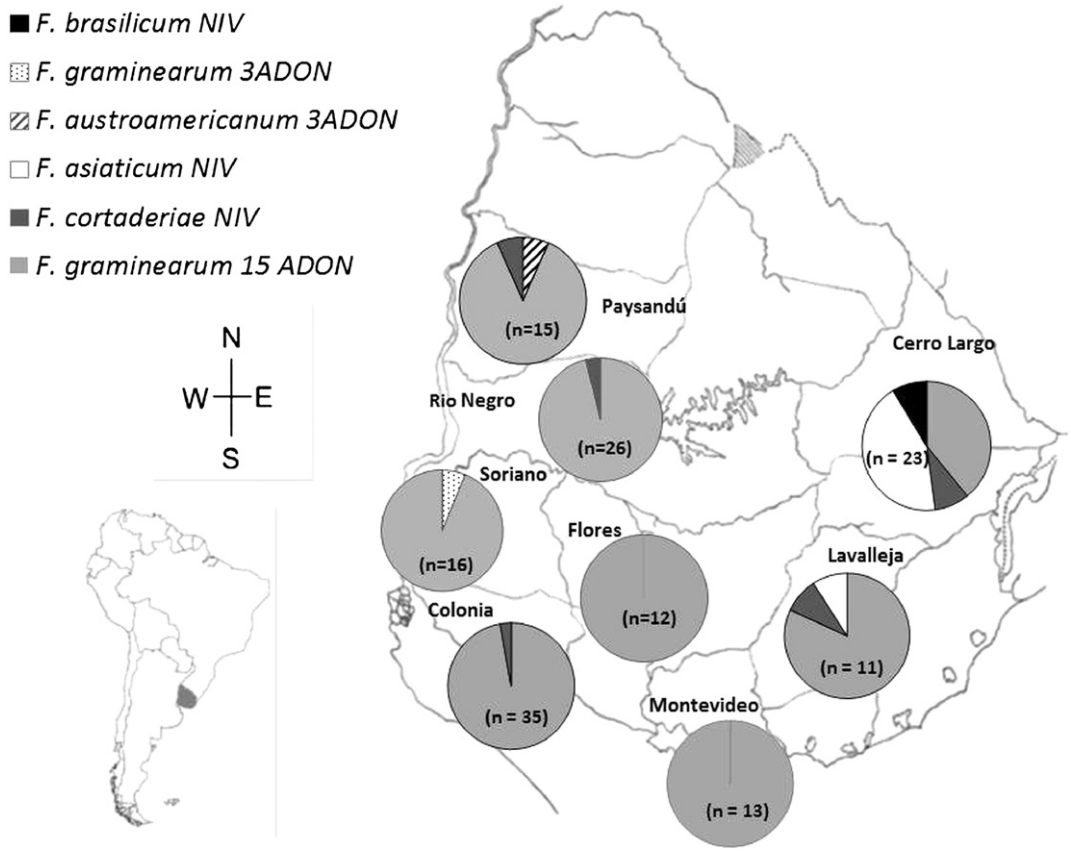

Fig. 1. Geographic distribution of FGSC species and trichothecene types in wheat grains in Uruguay.

addition, Lee et al. (2009) reported that *F. asiaticum* may be more fit in a rice agroecosystem than are the other species of the FGSC present in Korea. As such, it is possible that the predominance of *F. asiaticum* in wheat from parts of eastern Uruguay is the result of an introduced FHB pathogen population, and is connected to rice production in this region.

Trichothecene diversity among the isolates examined was partitioned geographically, strongly tied to species differences, and was substantially greater than previously reported among FGSC isolates from Uruguay. Previous studies have documented the predominance of *F. graminearum* and the 15ADON type among FHB isolates from wheat in Uruguay, Argentina, and Brazil (Alvarez et al., 2009; Pan et al., 2013; Scoz et al., 2009) The results reported here confirm the overall dominance of *F. graminearum* and the 15ADON type in Uruguay, but documented substantial regional differences in diversity and demonstrated that FGSC isolates with the NIV type predominated (61%) in the new wheat production area of Cerro Largo in eastern Uruguay. Pan et al. (2013) observed only 2 of 111 FGSC isolates (1.8%) from Uruguayan wheat with the NIV type. However, this previous analysis was limited to the major wheat production areas in western Uruguay, where 3.4% of the isolates examined in this study had the NIV type.

The relatively large percentage of FGSC isolates with the NIV trichothecene type identified in the current study indicates that there is a substantial risk of nivalenol contamination, particularly in wheat produced in eastern Uruguay along the border with Brazil. It is interesting to note that in a review of global data on mycotoxin levels in food and feed, Brazil was among a small number of countries in which nivalenol contamination levels were reported to be comparable with levels of DON contamination (Gale et al., 2011; Placinta et al., 1999). Nivalenol contamination of grain in Uruguay could have

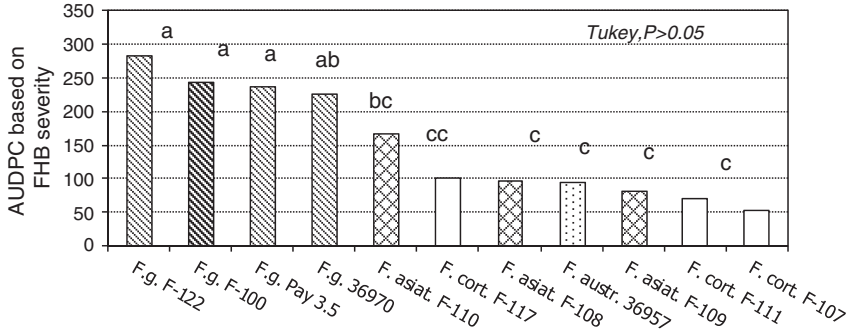

Fig. 2. Area under disease progress curve (AUDPC) based on FHB severities at 7, 14 and 21 days after inoculation of different *Fusarium graminearum* species isolated from wheat grains in 2002 and 2009. F.g.: *Fusarium graminearum*; F. asiat.: *F. asiaticum*; F. austr.: *F. austroamericanum*; F. cort.: *F. cortaderiae*.

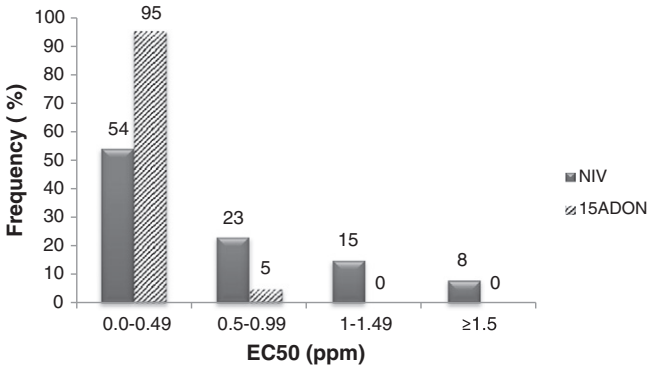

Fig. 3. Frequency distribution for the effective concentration of tebuconazole that results in 50% mycelial growth inhibition (EC50) for FGSC isolates from different locations in Uruguay. Full bars: isolates corresponding to the NIV chemotype; Striped bars: isolates corresponding to 15ADON chemotype.

significant implications for food safety and animal health because nivalenol appears to be more cytotoxic than DON (Luongo et al., 2008), and because nivalenol contamination is not currently regulated in Uruguay. The results reported here indicate the need for surveys to determine the prevalence and concentration of nivalenol in wheat and other grains from Uruguay. Additional surveys to determine the host or geographic distribution and movement of FGSC species and trichothecene types in Uruguay may also be warranted.

All of the FGSC species examined in our greenhouse experiments were capable of inducing FHB, although with different levels of aggressiveness, which is consistent with the results of previous studies indicating that all species within the FGSC can induce FHB on wheat (O'Donnell et al., 2000; Sarver et al., 2011; Starkey et al., 2007). However, isolates of *F. graminearum* were more aggressive on wheat than those of the other species examined. Even though there are several studies on the aggressiveness of *Fusarium* species, few reports have been published comparing the aggressiveness of species within the FGSC on wheat. O'Donnell et al. (2000) found no consistent differences in aggressiveness in an analysis of seven species within the FGSC. When comparing isolates of *F. graminearum* and *Fusarium boothii* from different geographic origins, Tóth et al. (2008) and Malihipour et al. (2012) found that *F. graminearum* had consistently higher aggressiveness in terms of FHB severity than *F. boothii* isolates. Gale et al. (2011) reported higher aggressiveness for DON-type isolates in comparison to NIV-type isolates, which is consistent with our findings in that the *F. graminearum* we tested were all of the 15ADON type, whereas the *F. asiaticum* and *F. cortaderiae* had the NIV type. However, the single *F. austroamericanum* strain that we tested had the 3ADON type and was significantly less aggressive than the *F. graminearum* strains with the 15ADON type, indicating that the relationship between DON or NIV and aggressiveness is not simple. Gale et al. (2011) also reported that *F. asiaticum* with the NIV type was more aggressive than *F. graminearum* with the NIV type, and concluded that toxin type can be a determinant of aggressiveness, but that aggressiveness is partially based on species and population-specific features. Again, our results support this conclusion, but systematic testing of many more isolates representing different species, populations, and toxin types within the FGSC is required to understand this relationship in more detail. The results of the current study also suggest that screening for FHB resistance may require the use of highly aggressive isolates or a mixture of isolates representative of the FGSC diversity present in Uruguay and neighboring countries.

Although FGSC isolates with the NIV type were less aggressive than *F. graminearum* with the 15ADON type, the NIV isolates we examined were found to be less sensitive to tebuconazole than 15ADON isolates. Again, the correlation between species and trichothecene type makes it difficult to determine if trichothecene type plays a direct role in this observation. None of the isolates that we examined would be resistant to tebuconazole as applied in field conditions based on the findings of Yin et al. (2009); however differences in sensitivity to tebuconazole may still have important effects on the composition of FHB pathogens over time. To our knowledge, this is the first analysis of tebuconazole sensitivity for *Fusarium* species causing FHB in wheat in Uruguay, and will be important for monitoring temporal or spatial changes in FGSC sensitivity to tebuconazole, which is critical to define FHB management practices.

5. Conclusion

We characterized for the first time the species and trichothecene diversity of *F. graminearum* species complex isolates responsible for FHB in traditional and new wheat production areas within Uruguay. This is the first report of FGSC species other than *F. graminearum* in Uruguay, and this study documents substantial variation in trichothecene type, aggressiveness, and tebuconazole sensitivity. Significantly, the results of this study indicate that *F. asiaticum* and *F. cortaderiae* with the NIV trichothecene type are major contributors to FHB of wheat grown in new production areas in eastern Uruguay. This finding is of significant concern to food safety and animal health and indicates the need for surveys to determine the prevalence and concentration of nivalenol in wheat and other grains from Uruguay. Analyses of nivalenol contamination in rice may be particularly important, as previous studies have suggested that *F. asiaticum* may be particularly well adapted to rice agroecosystems, and we have demonstrated the predominance of *F. asiaticum* and the NIV trichothecene type among wheat isolates from rice-growing regions in Uruguay.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2013.06.029.

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