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Morphological characterization and trichothecene genotype analysis of a Fusarium Head Blight population in South Africa

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Abstract Fusarium Head Blight is a major disease of wheat and an important contributor to the reduced cultivation of wheat in South Africa, where the crop often is grown under irrigation. We collected *Fusarium* isolates from 860 Fusarium Head Blight-infected wheat heads in seven irrigated wheat-growing areas of South Africa. Six *Fusarium* species, i.e., *F. chlamydosporum*, *F. crookwellense*, *F. culmorum*, *F. equiseti*, *F. graminearum* and *F. semitectum* were recovered, three of which, i.e., *F. chlamydosporum*, *F. equiseti* and *F. semitectum*, were not previously associated with Fusarium Head Blight in South Africa. *Fusarium graminearum* occurred at high frequencies at all seven locations. Based on polymerase chain reaction (PCR) assays of diagnostic sequences, more isolates were predicted to produce deoxynivalenol than nivalenol. *Fusarium graminearum* (sensu *lato*) appears to be the primary causal agent of Fusarium Head Blight in irrigated wheat in South Africa, which may not be the case for wheat cultivated under rain-fed conditions. Rotations of irrigated wheat with other graminaceous crops and maize could increase fungal inoculum and disease pressure. The establishment of Fusarium Head Blight in the irrigated wheat region of the country means that resistant lines and alternative agronomic practices are needed to limit disease severity, yield losses and mycotoxin contamination.

Keywords Deoxynivalenol (DON) · *Fusarium graminearum* · Mycotoxins · Nivalenol · Wheat scab

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Introduction

Fusarium Head Blight is one of the most devastating and intensively studied wheat diseases worldwide. Most recent work focuses on *F. graminearum* (sensu *lato*) with various trichothecene genotypes and genetic lineages dominating in different regions of the world (van der Lee et al. 2015).

At present in South Africa, wheat prices are low and production costs are high, with the import of lower quality wheat into the country further reducing the demand for locally grown wheat (Janeke 2014). Price, weather conditions and natural disasters are major contributing factors to the reduction in wheat acreage, but diseases, especially Fusarium Head Blight, which reduces both yield and grain quality, also can be a contributing factor.

Increased incidence of Fusarium Head Blight provides an additional economic disincentive for planting wheat and could be one of the reasons why South Africa has become a net importer of wheat.

Fusarium Head Blight is most severe when moist weather occurs during the flowering period in the growing season (Bai and Shaner 1994; McMullen et al. 1997). South Africa is unusual in that much of the wheat grown in the country is irrigated. Careful control of irrigation is required during this time to limit the conditions favorable for the development of Fusarium Head Blight. This disease not only reduces crop yield and seed quality, but also is associated with contamination of the grain by mycotoxins such as nivalenol, deoxynivalenol and zearalenone (Desjardins 2006; Leslie and Summerell 2006; McMullen et al. 1997).

Fusarium Head Blight may be caused by several species of *Fusarium*, with lineage 7 of *F. graminearum* (also known as *F. graminearum* sensu stricto) regarded as the primary causal agent in most geographic regions where the disease occurs. The fungus is homothallic (Leslie and Summerell 2006) with sexual development and the production of ascospores as inoculum thought to be a critical component of the disease cycle (Trail 2009). Based on morphological characters, i.e., spore and cultural characteristics, and biological characters, i.e., cross-fertility, *F. graminearum* is a single genetically diverse, widely distributed species (Leslie and Bowden 2008). If phylogenetic species descriptions are used, however, then *F. graminearum* can be subdivided into at least 15 species (O'Donnell et al. 2004, 2008; Sarver et al. 2011; Starkey et al. 2007; Yli-Mattila et al. 2009).

The objectives of this study were to identify the morphological species causing Fusarium Head Blight in the irrigated wheat-growing areas of South Africa and to determine the trichothecene production genotype(s) of the recovered isolates. We hypothesized that: i) *F. graminearum* (sensu *lato*) dominates in wheat afflicted with Fusarium Head Blight, and ii) that one of the deoxynivalenol genotypes will dominate within the *F. graminearum* population, as seen in wheat fields in other temperate wheat-growing regions. This study is the first evaluation of Fusarium Head Blight populations from irrigated wheat in South Africa, and differs significantly from reports of *Fusarium* populations from rain-fed crops grown in other parts of the country (Boutigny et al. 2011a).

Materials and methods

Field samples

Eight-hundred-and-sixty Fusarium Head Blight-infected wheat heads from commercial no-till wheat fields were collected from seven locations in the main irrigated wheat-growing regions in South Africa during two consecutive growing seasons (Fig. 1). Locations were selected based on past and present histories of Fusarium Head Blight epidemics. One-hundred-and-eighty wheat heads were collected at each of three locations (Prieska, Barkly West and Orania) that commonly report Fusarium Head Blight, and 80 wheat heads per site were collected at the other four locations, which do not regularly report Fusarium Head Blight. The grain samples were threshed manually.

Fungal isolation and identification

One *Fusarium* isolate was selected from each infected wheat head, even if multiple isolations could have been made. Seeds with Fusarium Head Blight symptoms were surface sterilized with 1.25 % (*v*/v) aqueous sodium hypochlorite solution for 3 min, rinsed three times for 30 s with sterile distilled water and dried at room temperature (20–25 °C) in a laminar flow hood. Surface-sterilized seeds were placed on a semi-selective medium for *Fusarium* (van Wyk et al. 1986) and incubated at 25 °C under a 12 h light/dark cycle for 4–7 days. After 4–7 days fungal hyphae were transferred to potato dextrose agar (PDA) [3.9 % (*w*/*v*) PDA bacteriological grade (BioLab®)] plates and incubated in a sterile growth chamber with a 12 h light/dark cycle at 25 °C for an additional 4–7 days. These fungal isolates were cultured on Spezieller Nährstoffarmer agar (SNA) (Nirenberg 1976) plates until conidia formed. Cultures originating from SNA plates were purified by subculturing single spores for identification purposes following dilution plating onto fresh PDA, SNA and carnation leaf agar (CLA) (Fisher et al. 1982; Leslie and Summerell 2006). All cultures used for morphological identification or trichothecene genotyping originated

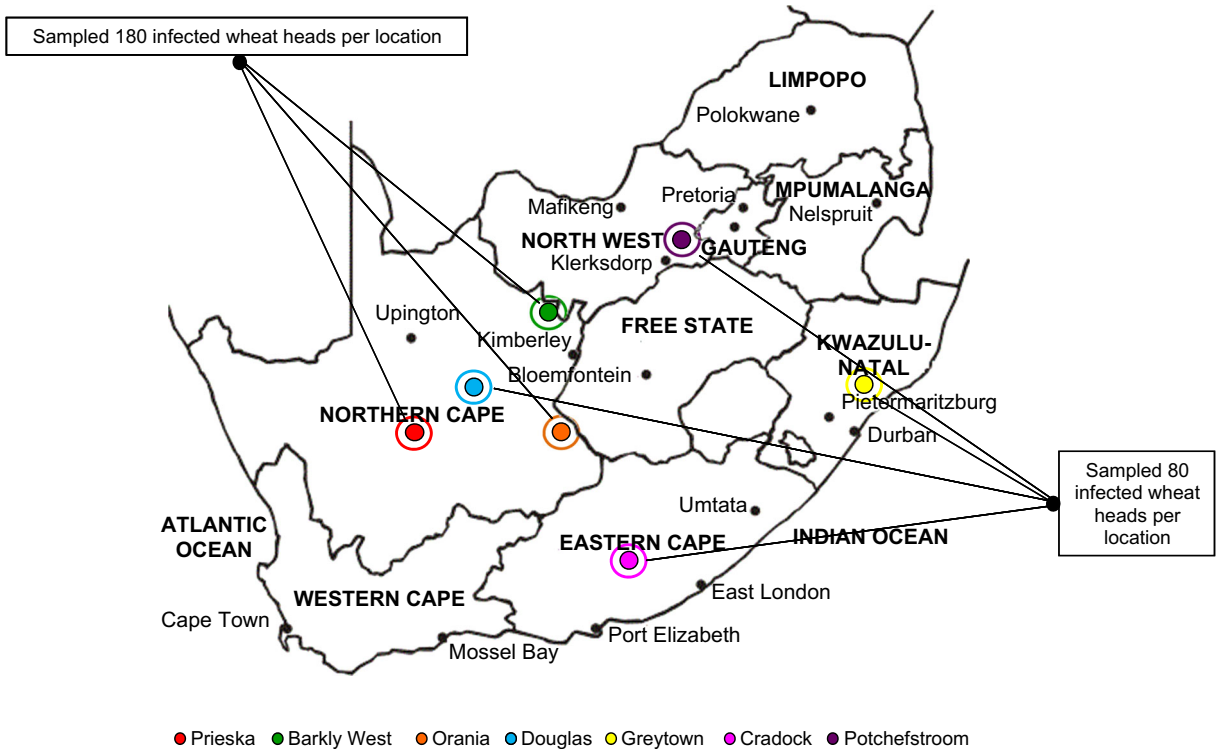

Fig. 1 Sampling locations in South Africa's main irrigated wheat-growing areas. The map delineates South Africa's nine provinces and identifies some of the major cities in each province

from a culture that grew from a single subcultured macroconidium. *Fusarium* species were identified morphologically based on the characters described by Leslie and Summerell (2006).

DNA extraction from fungal isolates

All isolates morphologically identified as *Fusarium* species were subcultured onto PDA plates and grown for 7–10 days at 25 °C. Mycelia were scraped from the PDA plates and freeze dried for three days at -60 °C with a Viritis Advantage Freeze Mobile II (New York City, New York, USA). The freeze-dried fungal material was ground to a fine powder with a Qiagen TissueLyser (Haan, Germany). Total genomic DNA was isolated by using a modified CTAB (hexadecyltrimethylammonium bromide) method (Herselman 2003). The quantity and quality of the DNA samples were determined by measuring absorbance at A_{260} and A_{280}. Samples were diluted with 1× TE buffer to a working concentration of 200 ng/μl for subsequent experiments.

Trichothecene genotype analyses

The trichothecene genotype of the *Fusarium* isolates was determined by PCR amplification of the *Tri7* and *Tri13* genes in the trichothecene biosynthetic gene cluster (Chandler et al. 2003). These genes encode proteins that convert deoxynivalenol to nivalenol (*Tri13*) and nivalenol to 4-acetyl-nivalenol (*Tri7*). The selected primer pairs can be used to identify deoxynivalenol (*DON*) and the nivalenol (*NIV*) genotypes (Tri7F + Tri7R and Tri13F + Tri13R) or just one genotype at a time (*DON*: Tri7F + Tri7*DON* and Tri13F + Tri13*DON*R; *NIV*: Tri7F + Tri7*NIV* and Tri13*NIV*F + Tri13R). Primer pair combination Tri13F + Tri13R targets the *Tri13* gene and can be used to identify both genotypes in a single reaction by amplifying a DNA fragment of 1075 base pairs (bp) associated with the *NIV* allele and/or a 799-bp fragment associated with the *DON* allele. Amplified fragment sizes between 458 and 535 bp resulting from amplification with the Tri7F + Tri7R primer pair amplification are associated with the *DON* genotype, while an amplified fragment size of 436 bp is associated with the *NIV* genotype.

PCR amplification reactions were performed in a total volume of 20 μl, containing ~1 μg of fungal genomic DNA, as described by Chandler et al. (2003). The optimized cycling conditions for the primers used were: one cycle at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 58 or 60 °C (depending on primer combination) for 30–45 s and 72 °C for 30 s followed by a final extension of 72 °C for 5 min and a 10 °C hold (Chandler et al. 2003).

Results

Morphological characterization

Based on morphological characterizations of 860 isolates, six *Fusarium* species, *F. chlamydosporum* (0.3 %), *F. crookwellense* (1 %), *F. culmorum* (2 %), *F. equiseti* (3 %), *F. graminearum* (93 %) and *F. semitectum* (0.7 %) were recovered. Three of these species, *F. crookwellense*, *F. culmorum* and *F. graminearum*, have previously been widely associated with Fusarium Head Blight. *Fusarium graminearum* was the dominant species associated with Fusarium Head Blight in South Africa and was present at high frequencies at all seven sampled locations (Table 1). The other two species known to cause Fusarium Head Blight, *F. crookwellense* and *F. culmorum*, were limited to Cradock, Greytown and Potchefstroom, in the less temperate wheat production regions (Fig. 1, Table 1).

Fusarium equiseti often is regarded as a saprophyte, but has been recovered at low frequencies from Fusarium Head Blight-infected grains in some European countries and Australia (Akinsanmi et al. 2004; Bottalico and Perrone 2002; Ioos et al. 2004). The highest percentage (6 %) of *F. equiseti* was found in the Barkly West field population, although this species was recovered at four of the seven sampled locations. Barkly West was the only location at which *F. chlamydosporum* (1 %) was recovered. The composition of *Fusarium* field populations at Prieska, Orania and Douglas were similar with only two species detected, *F. graminearum* and *F. equiseti*. The Prieska field population had the highest percentage (99 %) of *F. graminearum* isolates.

Four *Fusarium* species, *F. graminearum*, *F. culmorum*, *F. crookwellense* and *F. semitectum* were detected at Greytown, Potchefstroom and Cradock (Table 1). The Greytown field population had the highest observed frequencies for *F. crookwellense* (9 %) and *F. semitectum* (3 %). The Greytown and Potchefstroom field populations had the lowest percentage (84 %) of *F. graminearum* isolates. The Potchefstroom field population contained the highest frequency of *F. culmorum* (11 %). The lowest frequencies of *F. crookwellense* and *F. semitectum* were observed in the Cradock field population, but this population contained the second-highest frequency of *F. culmorum* (8 %).

Trichothecene genotype analysis

The trichothecene production genotype (*DON* or *NIV*) was determined for all isolates of *F. crookwellense*, *F. graminearum*, and *F. culmorum*, by using PCR assays. Trichothecene genotypes were not determined for isolates of *F. chlamydosporum* or *F. semitectum*. Most (89 %) of the *F. graminearum* isolates were *DON*. *Fusarium equiseti* isolates were ~50:50 *DON:NIV*. The *NIV* genotype was slightly favored (56 %) amongst the *F. culmorum* isolates. All of the *F. crookwellense* isolates were *NIV*, which is consistent with previous reports that isolates of *F. crookwellense* produce only nivalenol (Desjardins 2006).

Trichothecene genotype frequencies varied by location (Table 1). The potential for production of deoxynivalenol was highest at Prieska, where 99 % of the strains had the *DON* genotype. The *F. graminearum* populations at all locations had a higher frequency of *DON* (63–99 %) than of *NIV* alleles. There were more *DON* isolates than *NIV* isolates of *F. equiseti* at Prieska, Douglas and Orania, and more *NIV* isolates than *DON* isolates at Barkley West. For *F. culmorum*, there were more *DON* isolates at Cradock and Potchefstroom and more *NIV* isolates at Greytown.

Discussion

Fusarium Head Blight in South Africa

The first report of Fusarium Head Blight in South Africa was in 1980 in the North West Province along the Vaal River (Scott et al. 1988). The farm, in the Potchefstroom district (North West Province) from which we sampled infected wheat heads, also is located on the Vaal River. In 2006 Fusarium Head Blight was detected in this area for the first time since the original 1980 report.

Table 1 Frequency of deoxynivalenol (*DON*) and nivalenol (*NIV*) genotypes in various *Fusarium* species present in seven field populations of irrigated wheat in South Africa

Location Species	Number of isolates	Species frequency (%) per location	% DON	% NIV
Prieska	180		98	2
F. graminearum	178	99	99	1
F. equiseti	2	1	100	0
Barkly West	180		86	13
F. graminearum	168	93	89	11
F. equiseti	10	6	40	60
F. chlamydosporum*	2	1	0	0
Orania	180		89	11
F. graminearum	174	97	90	10
F. equiseti	6	3	67	33
Douglas	80		90	10
F. graminearum	76	95	91	9
F. equiseti	4	5	75	25
Greytown	80		71	25
F. graminearum	67	84	84	16
F. crookwellense	7	9	0	100
F. culmorum	3	4	33	67
F. semitectum	3	3	0*	0*
Cradock	80		84	15
F. graminearum	72	90	88	12
F. crookwellense	1	1	0	100
F. culmorum	6	8	67	33
F. semitectum	1	1	0*	0*
Potchefstroom	80		56	41
F. graminearum	67	84	63	37
F. crookwellense	2	3	0	100
F. culmorum	9	11	67	33
F. semitectum	2	3	0*	0*
Total Population	860		85	14
F. graminearum	802	93	89	11
F. chlamydosporum	2	0.3	0*	0*
F. crookwellense	10	1	0	100
F. culmorum	18	2	44	56
F. equiseti	22	3	50	50
F. semitectum	6	0.7	0*	0*

*Trichothecene genotype not determined

Fusarium Head Blight was epidemic in the early 1990s on farms along the Orange River in the Northern Cape where wheat is grown under center-pivot irrigation (Boshoff et al. 1999). Four of the seven locations (Prieska, Barkly West, Orania and Douglas) from which we collected are in this portion of the Northern Cape. Prieska, in 1991, was one of the first locations in the Northern Cape where Fusarium Head Blight was detected on wheat under center-pivot irrigation (Boshoff et al. 1999). Since 1991 epidemics of Fusarium Head Blight have continued in the irrigated wheat fields of the Northern Cape, with factors such as

tillage practices and a wheat/maize crop rotation as major contributing factors.

Elsewhere, Fusarium Head Blight outbreaks in KwaZulu-Natal were first reported in the 1985/86 season and included Greytown, one of the locations where we sampled. The most severe outbreaks in KwaZulu-Natal occurred near Winterton, an area where irrigated wheat often is rotated with maize in a long-standing maize production area. Follow-up reports of Fusarium Head Blight outbreaks near Greytown resumed in 2004 and reached epidemic status in 2006. In the Eastern Cape province, wheat farms are irrigated with water from the Great Fish River. Farmers in the Cradock district of the Eastern Cape were first plagued with Fusarium Head Blight in 2006.

Our results are consistent with previous reports that *F. graminearum* (sensu *lato*) is responsible for most of the outbreaks of Fusarium Head Blight in South Africa (Boshoff et al. 1999; Boutigny et al. 2011a; Scott et al. 1988). However, we focused on irrigated wheat, an unusual method for cultivating wheat, which usually is grown as a rainfed crop. The present study includes the bulk of the irrigated wheat-growing regions in South Africa and evaluated more isolates from more locations than did previous reports. Three of the six identified species, *F. crookwellense*, *F. culmorum* and *F. graminearum*, have been associated previously with Fusarium Head Blight in South Africa. These three species plus *F. equiseti* all have been associated previously with Fusarium Head Blight in other parts of the world (Scott et al. 1988; Snijders and Snijders and Perkowski 1990; Sutton 1982). The present report of *F. equiseti* associated with Fusarium Head Blight is the first of this association for South Africa. The available data do not suffice to claim that this fungus causes Fusarium Head Blight in South Africa as Koch's postulates have not been completed with the isolates that we recovered. *Fusarium equiseti* was recovered only in temperate regions in conjunction with *F. graminearum*.

Distribution of *Fusarium* species and toxin genotypes

The toxin genotypes are predictive of, but not guarantors of, mycotoxin production. Mycotoxin production depends on environmental conditions and the function of all of the enzymes in the biosynthetic pathway. The relative frequency of toxin genotypes varied somewhat by species and by location, with 85 % of the strains evaluated having a *DON* genotype and *15* % having a *NIV* genotype. From a food safety perspective, nivalenol is approximately ten fold more toxic to mammals than is deoxynivalenol (Desjardins 2006). If all of the strains produce toxins at a similar level, then the potential threat to food safety is probably larger from nivalenol than it is from deoxynivalenol, as strains with a *NIV* genotype compose more than 10 % of the total population.

The distribution of *Fusarium* species varied by location. Four locations – Prieska, Barkly West, Orania and Douglas – have a history of Fusarium Head Blight epidemics since the early 1990s. The wheat-growing areas of this region are all under center-pivot irrigation and most of the farms, including the ones where we sampled, follow a double-cropping rotation system that probably contributes to the high disease incidence observed. The no-till maize fields are hypothesized to provide the primary inoculum for Fusarium Head Blight on wheat grown under center-pivot irrigation, with warm day temperatures and somewhat cooler night temperatures encouraging sexual reproduction and the production of ascospores, which serve as the primary inoculum for wheat (Boshoff et al. 1999).

Fusarium graminearum was the dominant species in the Fusarium Head Blight population from the main irrigated wheat-growing areas in South Africa constituting 93 % of the isolates recovered. This finding is consistent with the results of a recent study of *F. graminearum* from multiple hosts in South Africa (Boutigny et al. 2011a). Those authors identified several morphologically indistinguishable genetic lineages of *F. graminearum* (sensu *lato*) in their studies – lineages 2 (*F. meridonale*), 3 (*F. boothii*), 5 (*F. acacia-mearnsii*), 7 (*F. graminearum* sensu stricto), 8 (*F. cortaderiae*) and 9 (*F. brasilicum*) (Leslie and Summerell 2006).

Two other crops, maize and barley, may alter the fungal communities responsible for Fusarium Head Blight of wheat. Isolates of *F. graminearum* lineages 3 and 7 have been reported from barley in South Africa (Boutigny et al. 2011a, b). With the exception of one strain from several hundred, all of the South African barley isolates produced deoxynivalenol rather than nivalenol.

The lineage to which most isolates of *F. graminearum* from maize in South Africa belonged varies based on the portion of the plant that was sampled. Isolates from lineage 7 were common on maize crowns and roots (Boutigny et al. 2011a; Lamprecht et al. 2011), but were not recovered at all from maize ears (Boutigny et al. 2011a). Isolates from lineages 2

and 3 were both recovered from maize roots (Boutigny et al. 2011a; Lamprecht et al. 2011), but only lineage 3 isolates were reported from maize ears (Boutigny et al. 2011a). The lack of isolates of lineage 3 in our large set of samples suggests that strains belonging to this lineage are unable to produce ascospore inoculum either in sufficient quantity or at the right time to cause major problems in irrigated wheat fields. Most isolates from maize produced deoxynivalenol, with isolates from lineages 2, 5 and 8 responsible for most of the nivalenol isolates identified in these previous collections. Given the dominance of lineage 3 isolates in the maize samples, more work is needed to confirm that maize field debris left in no-till fields is the inoculum source for Fusarium Head Blight of wheat (Schaafsma et al. 2005) in the locations we evaluated. The population composition of *F. graminearum* (sensu *lato*) varies by location (van der Lee et al. 2015) and it is possible that multiple genetic lineages/phylogenetic species could be coexisting in the irrigated wheat fields of South Africa.

Two other *Fusarium* species, i.e., *F. crookwellense* and *F. culmorum*, associated with Fusarium Head Blight, were restricted to less temperate regions of the country in our study. *Fusarium equiseti* and *F. poae* also have been recovered from barley in South Africa (Boutigny et al. 2011b). These results are consistent with previous hypotheses that *F. graminearum* dominates in temperate, humid areas of the world, whereas *F. culmorum* and *F. crookwellense* occupy the corresponding niche in cooler regions (Parry et al. 1995; Waalwijk et al. 2003

Fusarium graminearum dominated at all seven locations sampled in the present study. This geographically widespread domination suggests that a shift has occurred in the composition of the *Fusarium* populations that cause Fusarium Head Blight, especially in the less temperate irrigated regions. The frequency of *F. graminearum* also has increased relative to *F. culmorum* in several European countries (Brennan et al. 2005; Waalwijk et al. 2003) and has been attributed to increased maize production.

We also find that the frequency of *F. graminearum* has increased, but changes in cropping pattern, which appear relatively stable, seem unlikely to be the reason for the observed shift in South Africa. No-till farming systems are common in the surveyed areas and could increase Fusarium Head Blight incidence and severity, as the debris from a previously infected crop could serve as an inoculum source for subsequent crops. Rotation with maize has been suggested to increase disease incidence and severity in wheat, as maize is an excellent host for *F. graminearum*. The fungus can colonize the entire maize plant and also can cause economically important stalk and ear rots.

Spores of *F. graminearum* also may be carried long distances in the atmosphere (Schmale et al. 2006), but these spores alone probably do not suffice to induce a widespread epidemic, although they could be important on a local scale (Schmale et al. 2005). Countering this airborne spore dispersal requires regional changes in tillage and crop rotation practices to effect significant disease control. Even if atmospheric spore movement is insufficient to initiate a major epidemic, it could move enough spores for migration to mix local subpopulations and prevent them from becoming genetically isolated.

Cropping practices probably are the most important influence on Fusarium Head Blight in the cooler regions of the country, where temperature increases could change the spectrum of *Fusarium* species present, as has been hypothesized to occur in Europe (Waalwijk et al. 2003). The relatively low frequencies of *F. culmorum* and *F. crookwellense* identified in this study are consistent with the hypothesis that an increase in average temperature could be changing the species composition of the fungal community in South African wheat fields.

Fusarium Head Blight epidemics are accompanied by the accumulation of mycotoxins, which can have a detrimental effect on food and feed quality and safety. The trichothecene genotypes, *DON* and *NIV*, can be distinguished in these South African populations by using allele-specific PCR assays (Lee et al. 2001, 2002) for the *Tri7* and *Tri13* genes (Chandler et al. 2003). The *DON* genotype (85 %) dominated in populations of all *Fusarium* species evaluated in this study. The *DON* genotype was more common in the *F. graminearum* population (89 %) than it was in the *Fusarium* population as a whole. The high frequency of the *DON* genotype is consistent with selection for this genotype in these populations. Other studies of *F. graminearum* have found that deoxynivalenol is important in the host plant/pathogen interaction in cereal grains (Brown et al. 2001; Champeil et al. 2004; Desjardins et al. 1996), which could suffice to explain the observed differences in the frequencies of the *DON* and *NIV* genotypes. Twenty *DON* isolates from the Prieska population were tested in a preliminary study and all were highly aggressive on wheat (de Villiers 2009).

In summary, we found that *F. graminearum* is the dominant fungal species associated with Fusarium Head Blight in South African wheat fields under center-pivot irrigation. The *DON* genotype dominated in the individual populations and in the population as a whole, a result that is consistent with the hypothesis that isolates with the *DON* genotype are more aggressive pathogens on wheat than are those with the *NIV* genotype. The relatively constant composition of the various populations, at least in terms of species present and the frequency of the *DON* genotype, suggests that selection pressures are similar across all irrigated wheat regions in South Africa, and that there is sufficient movement of isolates between locations to keep the local communities heterogeneous in spite of significant geographic separation and climatic differences. The potential shift in pathogen community composition due to climate change will be important to monitor in the coming years.

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