Seasonal variation in mycoflora associated with asymptomatic maize grain from smallholder farms in two provinces of South Africa

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Abstract

Seed quality plays an important role in the establishment of healthy crop stands. The aim of this study was to identify the mycoflora associated with maize grain collected over two growing seasons, one experiencing severe drought, from smallholder farms in KwaZulu-Natal (KZN) and the Eastern Cape (EC). These are two adjacent provinces in South Africa with many maize-producing smallholder farmers. Asymptomatic maize ears were collected at harvest during the 2014/2015 and 2015/2016 maize growing seasons from farms located in Hlanganani (KZN), Ntabamhlophe (KZN), KwaNxamalala (KZN), Bizana (EC) and Tabankulu (EC). Maize grain was subjected to seed health tests using the agar plate method. The percentage incidence of fungal species isolated from maize grain was determined with species identities confirmed by ITS sequencing. Eleven fungal genera were identified with *Fusarium* species and *Stenocarpella maydis* the most prevalent. *Fusarium verticillioides*, *Fusarium graminearum* and *S. maydis* were isolated from all sites in both seasons. No fungal species exhibited a higher incidence in the drought season across all sites. *F. graminearum* and *S. maydis* had higher incidences in the wetter season at four and three sites, respectively. *F. verticillioides* had a greater incidence at the EC sites, particularly the coastal Bizana site. We conclude that local factors that affect the inoculum levels of each species, such as land preparation, previous crop, amount of debris from previous season, fertilizer application, and the micro-environment at field scale had a greater impact than the drought season on the population structure of ear-rot pathogens. The widespread presence of fungi that are potentially mycotoxin-producing in asymptomatic maize grain poses health risks to consumers and is worthy of further investigation.

Keywords: *Fusarium* spp., grain mycoflora, maize, seed quality, smallholder farmers, *Stenocarpella maydis*

1 Introduction

Maize is considered to be "life" in sub-Saharan Africa due to the significant role maize production plays in food security (Fisher *et al.*, 2015). Maize occupies more than 27 % of the cereal area in African countries such as Kenya, Malawi and Zimbabwe (Smale & Jayne, 2003; Smale *et al.*, 2011). Annual maize production in South Africa is estimated at 12.2 million tons (average for the three seasons 2013/2014; 2014/2015 and 2015/2016; Greyling & Pardey, 2019). Data from the Appendices provided by these authors indicate that 5 % of the production over this period is by smallholder farmers with average yields of 1.6 Mg ha^{-1}, whereas commercial farm yield was 4.4 Mg ha^{-1}. Kwazulu-Natal and Eastern Cape are two of the five main provinces

where maize is grown by smallholder farmers. Lower yields from smallholder farms have been ascribed to numerous abiotic and biotic stresses including low soil fertility, adverse environmental effects, weeds, pests and pathogens (Logrieco *et al.*, 2002; Jones & Thornton, 2003). Extreme environmental conditions, including drought, contribute significantly to maize yield reductions, especially in smallholder maize production systems, since more than 90 % of maize produced in South Africa is produced in rainfed areas (DAFF, 2012). This was illustrated by Jones & Thornton (2003) who, through the use of climate simulation models, predicted a yield reduction of 19 % by 2025 in South African smallholder rainfed maize farms.

Among biotic stresses, fungal pathogens account for 10-30 % of maize yield losses (Logrieco *et al.*, 2002). They cause three quarters of diseases associated with maize seed

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and grain (Cassini & Cott, 1979), and can be soil-borne, seed-borne or seed transmitted (McGee, 1988). Maize ears are prone to infection by *Fusarium* spp. which result in ear rot and infected grain. The most prevalent Fusarium spp. that cause ear rot in most maize growing regions are *Fusarium verticillioides* (Sacc) and *Fusarium graminearum*, also known as *Gibberella zeae* (Schw.) Petch (Munkvold, 2003). *Stenocarpella maydis* (Berk.) B. Sutton causes Diplodia ear rot, a devastating pre-harvest ear rot disease (Masango *et al.*, 2014). Several other pathogens such as *Alternaria* spp., *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Bipolaris* spp., *Cladosporium* spp., *Rhizopus* spp., *Colletotrichum* spp. and *Phoma* spp. have been associated with maize grain in South Africa (Basak & Lee, 2002; Somda *et al.*, 2008). Many of these pathogens produce secondary metabolites known as mycotoxins posing a serious threat to human and animal health (Meyer *et al.*, 2019).

The aim of the research was to determine the influence of seasonal variation on the mycoflora associated with hybrid maize grain from smallholder maize farms in the Eastern Cape and KwaZulu-Natal over two consecutive growing seasons. The main finding was that *Fusarium graminearum*, *F. verticillioides* and *S. maydis* were the most prevalent ear-rot pathogens at all five sites. Our data did not show consistent support for the hypothesis that the drought season would show a predominance of specific fungal species.

2 Materials and methods

2.1 Source of maize grain and site selection

Mixtures of maize grains from different hybrids (approximately 50 % were genetically modified with herbicide tolerance and/or insect resistance, but with not more than 25 % insect resistance) were obtained from five selected smallholder maize farms across KwaZulu-Natal and the Eastern Cape during the 2014/2015 and 2015/2016 summer seasons, defined as season 1 and season 2, respectively. All sites were part of smallholder farmer co-operatives and therefore there were several farmers involved at each site. Three of the farms were situated at Hlanganani, Ntabamhlophe and KwaNxamalala in KwaZulu-Natal Province of South Africa (Table 1). The other two sites were at Bizana and Tabankulu in the Eastern Cape Province (Table 1).

KwaZulu-Natal is characterized by moist grasslands with most of the rainfall occurring during summer months (October-February). KwaZulu-Natal has a subtropical climate which is characterised by high humidity and high temperatures in the summer months with some snow in the winter months in high altitude regions. The Eastern Cape also has its main rainfall during the summer months. It

has a highly variable climate with coastal regions experiencing frequent winds and high humidity compared to inland areas. A countrywide drought period and higher average daily temperatures were experienced during season 2 for both provinces as measured by the South African Weather Service (SAWS) (Rainfall and temperature data are provided Supplementary Fig. S1; Supplementary Table S1). The selected smallholder maize farms formed part of demonstration plots for seed company hybrids specifically developed for smallholder maize farmers in KwaZulu-Natal and the Eastern Cape. Farmers leading the demonstration plots, received seed of different hybrids from the seed company, as well as ad hoc support from extension officers during the season. The seeds were subjected to fungicide seed treatments with Celest® XL (Syngenta SA, South Africa) alone or in combination with other proprietary fungicide seed treatments before planting. Celest® XL is a fludioxonil and mefenoxam based fungicide. In South Africa, maize seeds are usually planted from mid-November until mid-December and is manually harvested from mid-May until the end of June (du Plessis, 2003). In the season 1 maize was planted from the end of October until mid-December and the season 2 maize was planted from the end of November until the beginning of January. This was due to a lower average rainfall at all planting sites in season 2 in comparison to the season 1.

2.2 Maize grain collection

Maize ears were collected from each maize field site at harvest in June 2015 and May 2016 for season 1 and 2, respectively. Maize at Bizana was at the growth stage R5 whilst the maize at other sites was at growth stage R6 when maize ears were collected. Approximately 20 maize ears were randomly collected per site across the five districts. The maize ears were placed in brown paper bags labelled with the date and district name. The ears were transported to the University of Pretoria Seed Science Laboratory where they were shelled. The grains from the 20 ears per site were combined and mixed thoroughly. Grains were sealed in airtight containers and stored at 4 °C until used.

2.3 Seed health test

Maize grain from each site were evaluated according to the modified International Seed Testing Association (ISTA) agar plate method (ISTA, 2013). Randomly selected maize grains collected at each site were surface sterilized in a 1 % sodium hypochlorite solution for five minutes. Two drops of Tween 20 (Sigma-Aldrich, Sigma-Aldrich, USA) were added to decrease surface tension on the grain surface. The maize grains were rinsed three times with sterile distilled

Table 1: *Sites in KwaZulu-Natal and Eastern Cape provinces of South Africa where maize grain was collected during season 1 and season 2.*

Community & closest town	Local Municipality	District Municipality	Province	Altitude (m asl)	Latitude	Longitude
Hlanganani	uMzimkhulu	Harry Gwala	KZN	1066	30°04'12.6"S	29°42'02.3"E
Ntabamhlophe	Inkosi Langalibalele	uThukela	KZN	1494	29°05'53.1"S	29°42'23.6"E
KwaNxamalala	uMngeni	uMgungundlovu	KZN	1145	29°36'29.2"S	30°13'27.9"E
Bizana	Mbizana	Alfred Nzo	EC	823	30°53'33.0"S	29°50'35.0"E
Tabankulu	Ntabankulu	Alfred Nzo	EC	941	30°53'33.9"S	29°31'37.1"E

water and air-dried on sterile blotter paper in a laminar flow. Ten grains per plate were aseptically plated on Petri dishes (9 cm diameter) containing half strength potato dextrose agar (PDA) (New England Biolabs, Ipswich, USA) amended with 0.05 g L^{-1} streptomycin (Sigma-Aldrich, Sigma-Aldrich, USA). The grains were incubated at 25 °C for seven days under blue/black light (365 nm) with alternating cycles of 16 h light and 8 h darkness to induce spore production by fungal cultures. Four replicates of 100 grains per site were used in the experiment and Petri dishes were arranged in a completely randomized design. The fungal cultures grown from the grains were isolated by transferring individual spores to half strength PDA amended with 0.05 g/L streptomycin using the single spore isolation technique. Cultures were incubated under blue/black light for seven days at 25 °C. Grains were evaluated four days after plating using a Zeiss (10-40x) microscope where colony colour and diameter was noted. A second evaluation after seven days identified fungal species based on spore morphology and arrangement using a Zeiss (40-100x) microscope.

2.4 Morphological identification of fungi isolated from maize grain

Morphological characteristics used in the identification of fungal cultures included spore shape and size, the presence or absence of micro- and macroconidia, chlamydospore formation, the arrangement of spores on conidiophores, septation of conidia, the colour of mycelium and spores as well as the presence and absence of aerial false heads. Books and reference material on the morphology of fungi by Nelson *et al.* (1981, 1983), Ellis & Ellis (1997), Mathur & Kongsdal (2003) and Leslie & Summerell (2006) were used to identify fungi based on morphological characteristics. Pure cultures, for subsequent molecular identification of each identified fungal species were obtained by sub-culturing spores onto PDA using the single spore isolation technique. Cultures were incubated at 25 °C under blue/black light with a photoperiod of 16/8 h for 7 d until DNA extraction. Fungal species isolated were stored in a 15 % glycerol solution at -80 °C

for future use. The incidence of each fungal species was calculated for each site using the formula derived from Kapindu *et al.* (1999) where the number of maize grains from which a particular fungal species was identified, divided by the total number of maize grains plated out for each site, expressed as a percentage.

2.5 Molecular identification of fungi isolated from maize grains

The DNA was extracted using a modified version of the cetyltrimethylammonium bromide (CTAB) method (Muller *et al.*, 2016). Liquid nitrogen was used to freeze-dry fungal material of each fungal species identified. Eighty to 100 mg of freeze dried material of each sample were mixed with 1 mL of a 2 % (w/v) CTAB solution containing 4 % (w/v) polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, USA), 0.5 % (v/v) β mercaptoethanol (Sigma-Aldrich, St. Louis, USA) and 2 μL RNAse A (7,000 U mL^{-1}) (QIAGEN, Hilden, Germany) and incubated at 37 °C for 30 min. After incubation, 1 μL of Proteinase K (600 mAU/mL) (QIAGEN, Hilden, Germany) was added to each sample followed by incubation at 65 °C for 60 min. Chloroform (1 mL) (Merck, Darmstadt, Germany) was added to each sample before centrifugation for 10 min at 10,000 rpm. Following centrifugation, the aqueous phase of each sample was transferred to new tubes and 0.08 volumes of 7.5 M ammonium acetate (New England Biolabs, Ipswich, USA) and 0.5 volumes of isopropanol (Merck, Darmstadt, Germany) was added to each sample. Samples were incubated at -20 °C for 24 h after which 1 mL of isopropanol was added to each sample and centrifuged at 10,000 rpm. After centrifugation supernatants were discarded and 70 % ethanol added to each sample. Following centrifugation at 10,000 rpm supernatants were discarded and DNA pellets re-suspended in 50 μL ddd-H2O. DNA concentration of each sample was determined by using a Nanodrop (Thermo Fisher Scientific, Wilmington, USA).

Primer sets ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the ribosomal DNA Internal Transcribed Spacer (ITS) gene region of the grain mycoflora as described in

Meisel *et al.* (2009) with modifications. The KAPA2G Robust HotStart ReadyMix Taq (Kapa Biosystems, Sigma-Aldrich, St. Louis, USA) was used in the PCR mastermix. The PCR samples were comprised of 6.2 μL KAPA2G Robust HotStart ReadyMix Taq, 0.6 μL of each primer and 5 μL of DNA for a total volume of 12.5 μL. Thermal cycling parameters included an initial denaturation at 95 °C for 5 min, followed by 34 cycles of denaturation at 96 °C for 30 sec, annealing at 55 °C for 30 sec and elongation at 72 °C for 1 min and 30 sec. A final elongation step was performed at 72 °C for 7 min.

Morphological identification was confirmed by Sanger cycle-sequencing of the ITS PCR products using primer ITS1 as described in Meisel *et al.* (2009). Fungal species were identified by comparing the ITS sequences obtained to sequences deposited in the National Center for Biotechnology Information (NCBI) database using the nucleotide Basic Local Alignment Search Tool (BLASTN).

2.6 Statistical design and analysis

The Statistical Analysis Software (SAS) version 9.3 program was used for statistical analysis (SAS, 2011). The PROC GLM test was done to detect significant statistical differences on arsine-transformed data of the percentage incidence of fungi. The values presented in the Table 3 are untransformed data. In order to test whether data meet the assumptions of ANOVA, data was tested for normality using the Shapiro-Wilk test. Levene's test was used on data which was not normally distributed to test for homogeneity of variances. Since no differences in the homogeneity of variances were found, one way ANOVA was performed. Means were separated using Fisher's Least Significant Difference test at $p = 0.05$.

3 Results

3.1 Climate data

Rainfall data showed that season 1 (2014/2015) had greater rainfall than season 2 (2015/2016) in both KwaZulu-Natal and Eastern Cape provinces (Supplementary Fig. S1A; Supplementary Table S1B). All three KwaZulu-Natal sites had 25–27 mm more rain for the October-February average in season 1 compared to season 2 (Supplementary Fig. S1A). The inland Eastern Cape site Tabankulu had 38 mm more rain for the October–February average in the first season, whereas the coastal site Bizana did not have reduced precipitation (Supplementary Fig. S1A). Furthermore, all sites had higher maximum average temperatures by 2–3 °C in season 2, except Bizana (Supplementary Fig. S1C).

Minimum temperatures were also greater in season 2 (Supplementary Fig. S1B). Season 2 was classified as a drought season across southern Africa since there was a major El Niño event at this time (Blamey *et al.*, 2018). Our averaging of the SAWS data confirms that this applied to the KwaZulu-Natal and Eastern Cape sites sampled in this study, except for the coastal Bizana site. The impact of the drought in season 2 on overall maize production in South Africa is illustrated by the drop in estimated maize production from 14,9 million tonnes (season 1) to 10.6 million tonnes (season 2) (reported in Appendix A of Greyling & Pardey, 2019).

3.2 Morphological identification of fungi isolated from maize grain

Although grains used were asymptomatic, a brown discolouration of the embryo was observed on some grains. Fungal cultures of *Stenocarpella maydis* were isolated from grains collected at all five sites across both growing seasons. When grown on PDA, *S. maydis* had a white fluffy appearance (Supplementary Fig. S2A). This fungal species had a rapid growth pattern compared to the other fungal cultures isolated. No spores were produced. Chains of 5-15 conidia of *Alternaria alternata* (Fr.) Keissl. were observed similar to the description by Mathur & Kongsdal (2003). Conidia were light brown and obclavate with the presence or absence of conical beaks at the tip (Supplementary Fig. S2B). Conidia differed in shape and size. Cultures of *A. alternata* grown on PDA, appeared as light to dark green or brown colonies with a distinct white fluffy growth. *Fusarium graminearum* Schwabe cultures were initially cream to light pink in colour. As the colony matured, the colour changed to a distinct dark pink colour with white fluffy mycelium (Supplementary Fig. S2C). Macroconidia produced were long, slender and elongated with 3-5 septa and tapered at both ends. (Supplementary Fig. S2D). *Fusarium subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas was isolated from maize grains collected from all of the sites except Ntabamhlophe in season 2. *F. subglutinans* appeared as light pink fungal growth with a fluffy appearance when grown on PDA (Supplementary Fig. S2E). Microconidia of *F. subglutinans* were arranged on aerial falseheads on monophialid or polyphialid hyphal branches and were oval in shape (Supplementary Fig. S2F). Macroconidia were septate and slender with a slight curvature. Cultures of *Fusarium verticillioides* grown on PDA appeared as light pink colonies (Supplementary Fig. S3A). Long chains of microconidia were displayed on monophialid hyphal branches (Supplementary Fig. S3B). Macroconidia of *F. verticillioides* had a distinct crescent shape and were 3-5 septate with tapered ends. *Sarocladium strictum* (W. Gams) Summerbell (Sup-

plementary Fig. S3C) was isolated from maize grains obtained from all five sites and both seasons except Bizana in season 2. When grown on PDA, *S. strictum* appeared yellow to light pink in colour. Conidia of *S. strictum* appeared as round wet clusters on mycelial tips (Supplementary Fig. S3D). *S. strictum* grew considerably slower compared to the other fungal species isolated. Additional fungal species were present at low incidence at only a few sites, and these were further identified by molecular analysis.
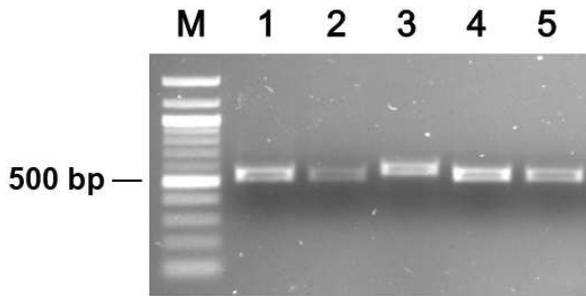
Fig. 1: *ITS1/4 region PCR amplification of DNA extracted from selected pure cultures of the main fungal species from grain mycoflora showing that DNA was of high quality prior to sequencing. Lane M, 100 bp molecular marker (Fermentas). DNA templates used in each lane: lanes 1-5, Fusarium subglutinans, Fusarium graminearum, Fusarium proliferatum, Stenocarpella maydis, Fusarium verticillioides.*

3.3 Molecular identification of fungi isolated from maize grains

The PCR amplification of the ITS gene region of single spore isolates resulted in amplification products ranging between 510–550 bp (Fig. 1). All the fungi molecularly identified from maize grains are shown in Table 2. Species belonging to eleven different fungal genera were identified from the collected maize grains. In addition to the dominant species, namely *Fusarium* spp., *S. maydis*, *S. strictum*, and *A. alternata*, the molecular analysis showed top BLASTN hits to *Chaetomium globosum*, *Cladosporium tenuissimum*, *Colletotrichum graminicola*, *Curvularia borreriae*, *Mucor fragilis*, *Nigrospora sphaerica*, *Nigrospora oryzeae*, *Penicillium cecidicola*, and *Sarocladium zeae*.

3.4 Distribution of fungi isolated from maize grains

3.4.1 Fungal species distribution per site

The most frequently isolated fungi were *F. graminearum*, *F. verticillioides* and *S. maydis* (Table 3 comparing % incidence values down each column, Fig. 2). The following species showed the highest incidence that was significantly greater than other fungal species: *F. graminearum* at
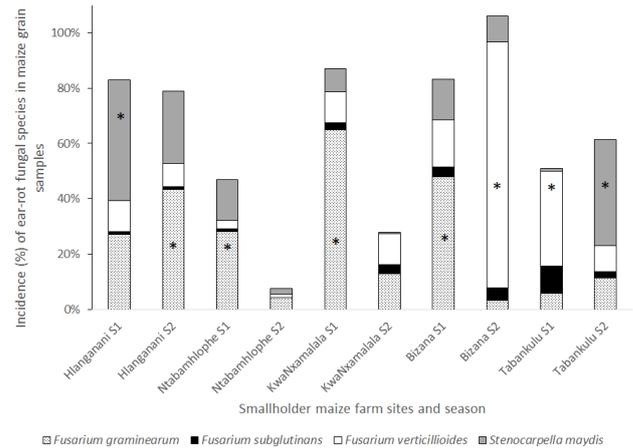
Fig. 2: *Incidence of the four main ear-rot fungal species at five sites over two seasons. The graph shows the average percent incidences of three Fusarium species and Stenocarpella maydis in four replicate samples of 100 maize kernels from three sites in KwaZulu-Natal (Hlanganani, Ntabamhlophe, KwaNxamalala) and two sites in Eastern Cape (Bizana, Tabankulu) in the first (S1)(2014/2015) and second (S2)(2015/2016) seasons. Drought conditions occurred in S2. Species that had significantly higher incidence than all other species in a sample are indicated with an asterisk (*)(Fishers LSD test; p < 0.05). Data was obtained from Table 3.*

Hlanganani-Season2 (S2) (43 %), Ntabamhlophe-S1 (28 %), KwaNxamalala-S1 (65 %), Bizana-S1 (48 %); *F. verticillioides* at Bizana-S2 (89 %); and *S. maydis* at Hlanganani-S1 (44 %) and Tabankulu-S2 (39 %) (Fig. 2, Table 3).

The KwaZulu-Natal sites had a prevalence of *S. maydis* and *F. graminearum* (Fig. 2, Table 3). Hlanganani maize grain samples were dominated by these two species with maximum incidence levels of 44 % (season 1) and 43 % (season 2), respectively. At Ntabamhlophe, *S. maydis* and *F. graminearum* were the main species in season 1 at 15 % and 28 %, respectively. At KwaNxamalala, *F. graminearum* was predominant in season 1 (65 %), but season 2 showed that *S. strictum* was most abundant (18.4 %). In the Eastern Cape, *F. verticillioides* reached 89 % incidence at Bizana and 34 % at Tabankulu, whereas it was present only up to 11 % at the KwaZulu-Natal sites.

A comparison of the three main *Fusarium* species revealed that *F. graminearum* was more prevalent at KwaZulu-Natal sites whereas *F. verticillioides* had high incidence at Eastern Cape sites (Fig. 2). *F. subglutinans* was present at low levels at most sites. *F. verticillioides* tended to be negatively correlated with *F. graminearum*, since samples with high *F. graminearum* incidence had low *F. verticillioides* content and vice versa (Fig. 2). *Alternaria alternata* and *S. strictum* were present at low levels in all samples, with *S. strictum* being the most abundant in only one sample

Table 2: *Molecular identification of fungi isolated from maize grains collected from smallholder maize farms in KwaZulu-Natal and Eastern Cape Provinces, South Africa in season 1 and 2.*

Fungi species*	Genbank Accession number[†]	Total score	E-value	Identity (%)
Alternaria alternata	MN989198.1	952	0	100
Chaetomium globosum	MN173145.1	968	0	100
Cladosporium tenuissimum	KY921927.1	929	0	100
Colletotrichum graminicola	EF187914.1	968	0	99
Curvularia borreriae	HE861848.1	1007	0	100
Fusarium graminearum	KT318586.1	994	0	99
Fusarium oxysporum	MF630986.1	813	0	100
Fusarium proliferatum	KP003948.1	815	0	100
Fusarium subglutinans	AB587009.1	924	0	100
Fusarium verticillioides	MK790049.1	929	0	100
Mucor fragilis	KX421450.1	1064	0	99
Nigrospora sphaerica	MN215810.1	909	0	100
Nigrospora oryzeae	MG661721.1	917	0	100
Penicillium cecidicola	DQ123648.1	1009	0	99
Sarocladium strictum	AY428789.1	946	0	100
Sarocladium zeae	KJ608087.1	961	0	99.81
Stenocarpella maydis	KP164561.1	972	0	100

* Fungal species with top BLASTN hit to ITS sequence from maize grain isolate in this study.
[†] Accession number of top BLASTN hit

Table 3: *Incidence (%) of fungal species isolated from maize grains collected in smallholder maize farms in KwaZulu-Natal and the Eastern Cape provinces in season 1 and season 2 in South Africa.*

	KwaZulu-Natal						Eastern Cape					
	Hlanganani		Ntabamhlophe		KwaNxamalala		Bizana		Tabankulu			
	S1*	S2	S1	S2	S1	S2	S1	S2	S1	S2	LSD	CV (%)
Alternaria alternata[†]	-	$6^{bc\ op}$	1^{abm}	$2^{ab\ mn}$	$0.3^{a\ m}$	$0.3^{a\ m}$	$3^{ab\ m}$	$0.3^{a\ m}$	$0.5^{a\ mn}$	$8^{c\ nop}$	0.05	163
Fusarium graminearum	$27^{b\ o}$	$43^{c\ r}$	$28^{b\ p}$	$4^{a\ n}$	$65^{d\ p}$	$13^{a\ n}$	$48^{c\ o}$	$3^{a\ m}$	$6^{a\ no}$	$11^{a\ p}$	0.12	33
Fusarium subglutinans	$1^{ab\ m}$	$1^{ab\ mn}$	$1^{ab\ m}$	-	$3^{ab\ m}$	$3^{ab\ m}$	$4^{ab\ m}$	$5^{b\ m}$	$10^{c\ o}$	$2^{ab\ mn}$	0.04	89
Fusarium verticillioides	$11^{bc\ n}$	$9^{abc\ p}$	$3^{ab\ mn}$	$1^{a\ mn}$	$11^{bc\ o}$	$11^{bc\ n}$	$17^{c\ n}$	$89^{e\ o}$	$34^{d\ p}$	$9^{abc\ op}$	0.09	30
Penicillium cecidicola	-	-	$1^{a\ m}$	$5^{a\ n}$	$3^{a\ m}$	$4^{a\ m}$	$0.3^{a\ m}$	$1^{a\ m}$	47^{b} q	-	0.06	65
Rhizopus spp.	$2^{a\ m}$	$0.3^{a\ m}$	$1^{a\ m}$	$34^{b\ o}$	-	-	-	$3^{a\ m}$	$2^{a\ mn}$	-	0.04	69
Sarocladium strictum	$1^{ab\ m}$	$4^{ab\ mno}$	$8^{c\ n}$	$1^{ab\ mn}$	$3^{ab\ m}$	$18^{d\ o}$	$5^{bc\ m}$	-	$0.3^{a\ mn}$	$3^{ab\ mno}$	0.04	66
Stenocapella maydis	44d ^{p}	$26^{c\ q}$	$15^{b\ o}$	$2^{a\ mn}$	$8^{ab\ n}$	$0.5^{a\ m}$	$15^{b\ n}$	$10^{ab\ n}$	$1^{a\ mn}$	$39^{d\ q}$	0.11	47
LSD	0.09	0.05	0.06	0.04	0.03	0.04	0.05	0.05	0.06	0.07		
CV (%)	93	61	122	104	43	113	69	57	73	123		

* Means in each column (m-r) with the same letter are not significantly different according to Fisher's LSD test ($p < 0.05$). S1 = season 1 (2014/2015); S2 = season 2 (2015/2016).
[†] Means in each row (a-e) with the same letter are not significantly different according to Fisher's LSD test ($p < 0.05$).

(KwaNxamalala-S2)(Table 3). *P. cecidicola* and *Rhizopus* spp. were not present in all samples, and had the highest incidence in one sample each only (*P. cecidicola*: 47 % at Tabakulu-S1; *Rhizopus* sp.: 34 % at Ntabamhlophe-S2) (Table 3).

3.4.2 Seasonal variation of fungi isolated from maize grains

There was significant variation in fungal species incidence between sites and seasons (Table 3, comparing % incidence values across each row). *F. graminearum* incidence was significantly different between season 1 and season 2 at all sites,

except Tabankulu (Table 3, Fig. 2). *F. graminearum* levels were higher in season 1 at Ntabamhlophe, KwaNxamalala and Bizana. This species was present at relatively high levels in both seasons at Hlanganani (27 % and 43 %), but incidence was greater in season 2. *F. verticillioides* was present in all samples, but only significantly different between seasons at the Eastern Cape sites. Levels of *F. verticillioides* decreased from season 1 (34 %) to the drought season 2 (9 %) at Tabankulu, whereas at Bizana, which did not experience drought, the incidence was greater in season 2 (89 %) (Table 3, Fig. 2). *F. subglutinans* was present at all sites but levels were only significantly different at Tabankulu, where this species was significantly less in the drought season 2 (Table 2). *S. maydis* had a greater incidence in season 1 at all KwaZulu-Natal sites and Bizana, although the difference was not statistically significant at the latter site and KwaNxamalala. At Tabankulu, the opposite was seen with significantly greater *S. maydis* incidence in season 2 (Table 3, Fig. 2). *A. alternata* and *S. strictum* did not show consistent variation over seasons. There were significantly greater incidences in drought season 2 of *A. alternata* at Hlanganani and Tabankulu, and *S. strictum* at KwaNxamalala (Table 3).

4 Discussion

This study determined the seasonal mycoflora associated with hybrid maize grain from smallholder maize farms in the Eastern Cape and KwaZulu-Natal over two consecutive growing seasons with the second season experiencing severe drought. From the current study eleven genera of fungal species were identified. The most frequently isolated fungi per site in order of prevalence were *F. graminearum*, *F. verticillioides*, *S. maydis*. *P. cecidicola*, *S. strictum*, *Rhizopus* spp., *F. subglutinans* and *A. alternata*. All the genera identified using molecular analysis namely *Fusarium, Stenocarpella, Alternaria, Chaetomium, Cladosporium, Colletotrichum, Curvularia, Mucor, Nigrospora, Penicillium* and *Sarocladium*, have all been recorded on maize grain (Warham *et al.*, 1996; Saunders & Kohn, 2008; Somda *et al.*, 2008; Abe *et al.*, 2015). *Colletotrichum graminicola* has been recorded to cause red stalk rot of cereals in South Africa (CABI, 2020). The high incidence of *P. cecidicola* (47 %) at Tabankulu in season 1 was probably due to the close proximity of the pig stiles to the maize field. *P. cecidicola* is a common mould causing food spoilage (Kawaguchi *et al.*, 2019), which raises the possibility of the source being food waste fed to the pigs. This fungus has been reported to cause preharvest wet core rot of apples in South Africa (van der Walt *et al.*, 2010). It is exceptionally resistant to UV light, requir-

ing more than 200 mJ cm^{-2} of radiation to kill the fungus (Kawaguchi *et al.*, 2019).

Fusarium and *Stenocarpella* genera had the highest incidence across all sites. In general the incidence of the isolated pathogenic species was higher in season 1 than season 2. This was found with *F. graminearum* in Bizana and Ntabamhlophe, *F. subglutinans*, *F. verticillioides* and *P. cecidicola* in Tabankulu, *Sarocladium* in Bizana and Ntabamhlophe and *Stenocarpella* in Hlanganani and Ntabamhlophe. However, exceptions included *F. graminearum* in Hlanganani, *F. subglutinans* and *F. verticillioides* in Bizana, *Sarocladium* in KwaNxamalala and *Stenocarpella* in Tabankulu where incidence was higher in season 2. There was also a proliferation in season 2 of *A. alternata* in Hlanganani and Tabankulu and *Rhizopus* in Ntabamhlophe. The incidence of the different fungal species is probably associated with drought and heat stress associated differences in prevailing weather conditions in the different geographic locations over the two growing seasons. Higher average daily temperatures were observed during October to December 2015 and from January to April 2016 at all the sites in this study (Supplementary Table S1) except Ntabamhlophe, KwaNxamalala and Tabankulu in January 2016. Minimum temperatures followed the same trends except at Tabankulu in November and January 2016 when temperatures were lower than the 2014/2015 growing season (season 1) (Supplementary Table S1). Temperatures on the African continent in 2015 marked the second warmest year other than 2010 as reported by the SAWS and the National Oceanic and Atmospheric Administration.

Ncube *et al.* (2011) reported that *F. subglutinans* was the dominant *Fusarium* spp. identified from maize grain collected from the Eastern Cape and Mpumalanga whilst *F. verticillioides* was the dominant fungal species isolated from Zululand and Limpopo. The results from the current study indicates that *F. graminearum* was the dominant *Fusarium* spp. isolated from KwaZulu-Natal whilst *F. verticillioides* was the most dominant *Fusarium* spp. isolated from the Eastern Cape. It has been reported that the frequency of *F. graminearum* varies significantly among years and locations in many geographical regions (Cook & Christensen 1976; Logrieco *et al.*, 2002; Boutigny *et al.*, 2011; Czembor *et al.*, 2015).

According to Cook & Christensen (1976) and Czembor *et al.* (2015), *F. verticillioides* germinates and grows over a broader range of temperatures and water activities than *F. graminearum* (minimum of 4 °C and 0.86 aw and 10 °C and 0.935 aw, respectively). Thus, *F. verticillioides* has the ability to eventually out-compete *F. graminearum* when both pathogens are present (Czembor *et al.*, 2015). The higher

incidence of *F. verticillioides* at Bizana in season 2 can be attributed to the later planting date (planting was delayed by one month from the end of November until the beginning of January) and the increased presence of maize stalkborer (incidence not recorded in this study). However, our sampling strategy from only asymptomatic maize ears reduced the probability of isolating from grain contaminated with fungi through insect damage. The number of rainy days has a positive relationship to spore production by *F. verticillioides* and rainfall and splashing favour spore dispersal (Rossi *et al.*, 2009). A study by Parsons & Munkvold (2010) also reported a higher incidence of *F. verticillioides* and insect populations (thrips and ear worm) on maize with a delayed planting date. Furthermore, the later planting date would have resulted in a higher moisture content of the maize grains at the time of harvesting compared to that of the maize grains from the other sites.

Stenocarpella maydis, the causal agent of Diplodia ear rot, is associated with stalk rot of maize (Gatch & Munkvold, 2002; Lamprecht *et al.*, 2013). *S. maydis* was more prevalent during season 1 than season 2 in KwaZulu-Natal probably as this pathogen prefers cooler temperatures and wet weather and conidia rapidly lose their viability at high temperatures and on exposure to sunlight (Lamprecht *et al.*, 2013).

Apart from rainfall and temperature, the variability in the incidence of fungal species, identified across the sites and years, could also be due to differences in: land preparation (extent of ploughing); previous season's crop; crop growth stages; row establishment; extent of weeding affecting the micro-climate at field scale; the amount of debris on the soil surface; and timing, type and amount of fertiliser applied (Bateman & Coşkun, 1995; Swer *et al.*, 2011). The composition of soil-borne mycoflora changes as the maize plant grows since some fungi are better competitors than other fungi (Schoeman & Craven, 2013). Soil conditions also play a role in the fungal diversity prevalent in the soil. Bateman & Murray (2001) reported that *Fusarium* spp. populations associated with wheat crops decreased with decreasing temperatures and increasing soil moisture. This can provide a possible explanation as to why differences in the fungal diversity where found across sites since the planting dates differed across sites. The mycoflora identified in the current study can either originate from direct infection of maize ears through the silks or wounds made by insects or birds, or via systemic transmission from the soil and seed through the plant to the maize ears (McGee 1988; Munkvold *et al.*, 1997; Somda *et al.*, 2008).

Systemic transmission of some of these fungi is still debated. Oren *et al.* (2003) observed the systemic transmission of *F. verticillioides*, through the use of green fluorescent protein, in early growth stages of maize within the mesocotyl and main roots although other tissues appeared asymptomatic. Basak & Lee (2002) reported systemic transmission of *F. verticillioides* and *A. alternata* from maize seeds to seedlings resulting in pre- and post-emergence death on maize in Korea. Systemic transmission of *S. maydis* was also reported from artificially infected maize seeds to plants by Siqueira *et al.* (2016). The transmission of *F. verticillioides* through the maize silk channel has been reported by Munkvold *et al.* (1997) and Duncan & Howard (2010). The high incidence of *F. verticillioides*, *F. graminearum* and *S. maydis* in each site could indicate a high tendency of these organisms to systemically colonize maize plants compared to the other fungi isolated (Mukanga *et al.*, 2010), due to their highly competitive nature. Although the grains were seemingly healthy, these pathogens were still isolated from them.

The most frequently isolated fungi per site in this study were *F. graminearum*, *F. verticillioides* and *S. maydis*. The change in environmental conditions from season 1 to season 2 maize growing seasons, with lower rainfall and higher temperatures, did not seem to favour any one particular pathogen among the studied regions. We conclude that local site-specific factors influencing fungal inoculum levels may be more important drivers of ear-rot species dynamics in these farming systems. These factors, that were not assessed in the current study, include prior inoculum in debris and the soil, and the extent of conservation agriculture practises, such as crop rotation, tillage levels, weed control and fertilizer applications (inorganic vs organic).

5   Conclusions and recommendations

Stenocarpella maydis and *Fusarium* spp., specifically *F. verticillioides* and *F. graminearum* had a high prevalence in maize grains collected from smallholder farms. As these pathogens can produce secondary metabolites of serious threat to human and animal health (Meyer *et al.*, 2019), mycotoxin analyses should be included to ensure food safety for the farmers. The high diversity of fungi prevalent in asymptomatic maize grains could give rise to latent infections. Further studies regarding the soil mycoflora need to be conducted in order to determine which of the grain mycoflora are transmitted from the soil to the plant and seeds. Such studies should also be conducted in a wider geographical range. A more comprehensive approach would be to do a fungal metagenomics analyses of grain samples as was done for the bacterial endophytes associated with Bt maize from South Africa by Mashiane *et al.* (2017). Future studies could also factor in the hybrid effect to identify the role that

each hybrid plays on seed mycoflora. This will in turn help to provide farmers with the best advice regarding prevention and control of diseases brought about by such fungi by recommending the best hybrids to plant within a given geographical range.

Supplement

The supplement related to this article is available online on the same landing page at: https://doi.org/10.17170/kobra-2020xvfr .

Figure S1. Summary of weather data. A: Average rainfall (Oct-Feb) for season 1 and 2 at the five sites. B: Average daily minimum temperature (°C) at each site for each season (Oct-April): e.g. B1, B2 = Bizana season 1 & 2, respectively. C: Average daily maximum temperatures for each season (Oct-April). Data from Supplementary Table S1.

Figure S2: Morphological characteristics of four fungal species associated with maize grains. (A) Culture of *Stenocarpella maydis*, (B) Conidia of *Alternaria alternata*, (C) Culture of *Fusarium graminearum*, (D) Chlamydospore of *F. graminearum*, (E) Culture of *Fusarium subglutinans*, (F) Conidia of *F. subglutinans*. All microscopic images were captured at 40x magnification.

Figure S3: Morphological characteristics of three fungal species isolated from maize grains. (A) Culture of *Fusarium verticillioides*, (B) Microconidia in chains on hyphae of *F. verticillioides*, (C) Culture of *Sarocladium strictum*, (D) Conidia of *S. strictum*. All microscopic pictures were captured at 40x magnification.

Table S1A. Average minimum and maximum temperature at sites in KwaZulu-Natal and Eastern Cape provinces of South Africa during the 2014/2015 and 2015/2016 maize growing seasons, named season 1 and season 2, respectively.

Supplementary Table S1B. Monthly rainfall at sites in KwaZulu-Natal and Eastern Cape provinces of South Africa during the 2014/2015 and 2015/2016 maize growing seasons, named season 1 and season 2, respectively.

Conflict of interest

The authors declare that they have no conflict of interest.

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