ORIGINAL RESEARCH

Fumonisin producing *Fusarium* spp. and fumonisin contamination in commercial South African maize

**B. Janse van Rensburg · N. W. McLaren ·
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Abstract Fumonisins are secondary, carcinogenic metabolites produced primarily by *Fusarium verticillioides* and *Fusarium proliferatum* on maize worldwide. The natural occurrence of fumonisin-producing *Fusarium* spp. and fumonisin contamination of maize grain were quantified in selected maize cultivars from principal production areas of South Africa. Grain colonization by *Fusarium* spp. was determined using quantitative real-time PCR (qPCR) and contamination with fumonisins using HPLC analysis. Kernels from the 2007 samples were also plated onto *Fusarium* selective medium and subsequently, split plates containing PDA & CLA. The number of fumonisin producing *Fusarium* spp. were quantified and microscopically identified after 14 days. Simple linear regression analysis was used to determine the relationship between target DNA, fumonisins and the number of fumonisin producing *Fusarium* spp. using the plating out method. Results indicated high natural infection by fumonisin-producing *Fusarium* spp. and fumonisin concentrations in warmer production areas such as Northern Cape, North West and Free State Provinces. Spearman Ranking Correlations indicated that the responses of cultivars to colonization of grain by fumonisin producing *Fusarium* spp. varied over localities/seasons (r_s=0.42 to 0.64) suggesting that cultivars reacted differently to different environmental/inoculum conditions (disease potentials). Cultivars CRN3505 and DKC80-12B showed a degree of resistance to fungal infection. As with fungal colonization, Spearman Rank Correlations indicated the response of cultivars to fumonisin contamination to vary over localities/seasons (r_s=0.29 to 0.70). Cultivars DKC80-12B and LS8521B showed a degree of resistance to fumonisin contamination. Regression analysis yielded a significant relationship between HPLC data and qPCR, but not with the plating out of grain data suggesting the former to be a better indicator of potential fumonisin contamination. Site-specific, daily maximum temperature and rainfall data were provided by the ARC-Institute for Soil Water and Climate's meteorology office. No significant relationship between these weather parameters and colonization of grain by fumonisin producing *Fusarium* spp. was recorded, although a tendency was observed between fumonisin contamination and mean maximum temperature.

Keywords Fumonisins · *Fusarium* spp · G X E interactions · Incidence

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Introduction

Maize (*Zea mays* L.) is an important crop in South Africa and is produced throughout the country under diverse cultural and weather conditions. The important

fumonisin-producing ear rot *Fusarium* spp. are *F. verticillioides* and *F. proliferatum* (Rheeder et al. 1990). The distribution and predominance of these *Fusarium* spp. and their concomitant fumonisin production varies depending on season, geographic locality, climatic factors such as temperature and moisture, host genotype and agricultural practices (Nyaka et al. 2010). At least 28 fumonisin analogues are known, but the most abundant natural forms are fumonisin B_1, B_2 and B_3 (Rheeder et al. 2002).

Fumonisins are secondary carcinogenic metabolites, which occur naturally as contaminants of agricultural products such as maize. The consumption of maize contaminated with fumonisins causes mycotoxicoses in animals including leucoencephalomalacia in horses (Kellerman et al. 1990; Ross et al. 1990) and pulmonary edema in swine (Harrison et al. 1990). Fumonisin infected maize has been statistically associated with human esophageal cancer in South Africa (Marasas 1981, 1982, 1988; Rheeder et al. 1992), northern Italy (Franseschi et al. 1990) and Iran (Shephard et al. 2000). Chu and Li (1994) and Li et al. (2001) reported an increased incidence of primary liver cancer in humans that ingest maize contaminated with fumonisin in certain regions of The People's Republic of China. Stack (1998), Placinta et al. (1999), Hendricks (1999) and Marasas et al. (2004) have shown a strong correlation between the consumption of fumonisin-contaminated tortillas and neural-tube defects in humans. The potential carcinogenic risk of fumonisin B_1 to humans was evaluated and classified by the World Health Organizations International Agency for Research on Cancer (WHO-IARC) as a "Group 2B carcinogen" which means it is possibly carcinogenic to humans (1993).

The United States Food and Drug Administration (FDA) has set guidelines of 2 ppm (FB_1) for degermed dry milled maize products and 4 ppm for whole or partially de-germed dry milled maize products for human consumption (FDA 2001). Currently South Africa has no legislation or monitoring system regarding allowable fumonisin concentrations and consumers may be at greater risk due to the higher consumption of maize in comparison to European countries (Marasas 2001). Marasas (2001) recorded large variations in probable daily intake (PDI) of maize ranging from 1.2 μg/kg bodyweight (bw)/day in urban South Africans consuming commercial maize, to 354.9 μg/kg bw/day in rural South Africans consuming moldy, home-grown maize.

Limited data are available regarding the incidence of *Fusarium* spp. and associated fumonisin levels in maize in South African production areas. Fumonisins in maize produced by subsistence farmers in northern KwaZulu-Natal (Zululand) province of South Africa exceeded 2 ppm set by the United States Food and Drug Administration in 52 % and 17 % of samples collected in 2006 and 2007 respectively (Ncube et al. 2011). These authors also reported a number of samples from Mokopane (Limpopo) and Lusikisiki (Eastern Cape) containing excessive fumonisin levels. Boutigny et al. (2012) reported that *F. verticillioides* was the predominant fungus in maize at 14 commercial localities in the North-West, the western Free-State and Northern Cape provinces, while *F. proliferatum* was not detected at any of the localities.

The objectives of this study were: (i) to quantify the incidence of fumonisin producing *Fusarium* spp. and to determine the concentrations of fumonisin in commercial maize grain samples from different production localities in South Africa, (ii) to study genotype x environment interactions associated with colonization and fumonisin contamination, (iii) to determine the relationship between fumonisin-producing *Fusarium* spp. with morphologically based identifications based on plating-out and fumonisin concentrations, and (iv) to evaluate the relationship between maximum temperature and rainfall and the contamination of grain by fumonisin-producing *Fusarium* spp. and resultant fumonisin contamination.

Materials and methods

Maize samples

Maize kernels harvested from National Cultivar Evaluation Trials conducted by the ARC-Grain Crops Institute in Potchefstroom were collected from a range of localities (Tables 1, 2, and 3) during the 2007–2009 maize production seasons. All trials were conducted using a randomized complete block design with three replicates. Trials were conducted under dry land conditions and maintained according to "Best Practice" appropriate to the respective production areas with the exception of Vaalharts in the Northern Cape which was flood irrigated. Weather variables, including daily maximum temperature and rainfall were monitored at each locality during flowering and grain development

Table 1 Mean fumonisin concentration using HPLC analysis, fumonisin-producing target DNA using qPCR, rainfall and maximum temperature during the 2007 maize production season

2007	DKC80-12B	DKC 78-15B	PAN 6611	LS 8521B	CRN 3505	DkC 80-10	Mean	DKC80-12B	DKC 78-15B	Pan 6611	LS 8521B	CRN 3505	DkC 80-10	Mean	Mean rainfall, Jan, Feb, Mar	Mean max temp, Feb, Mar	Mean max temp, Mar
	Fumonisin concentration (ppm)							Target DNA (pg fungal DNA/0.5 g milled maize kernels)									
Bainsvlei	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	15.30	nd	2.55[abc]	0.74	30.78	28.87
Bethlehem	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	0.93	28.24	27.18
Bloekomspruit	nd	nd	nd	nd	nd	0.04	0.00[a]	nd	nd	nd	1.47	2.02	nd	0.58[a]	1.48	28.49	27.98
Bothaville	nd	nd	8.84	nd	2.30	nd	0.67[b]	nd	nd	nd	nd	4.66	nd	0.77[a]	1.07	31.15	29.84
Delareyville	2.30	nd	nd	1.73	nd	nd	0.67[a]	7.31	nd	nd	nd	5.18	nd	2.08[abc]	1.22	30.65	29.33
Hartebeesfontein	nd	5.30	0.06	nd	nd	nd	0.89[a]	21.30	199.00	17.60	nd	nd	nd	39.65[de]	1.36	29.67	28.43
Koster	nd	15.24	1.56	nd	0.12	2.00	3.15[a]	2.11	23.80	9.98	nd	33.00	10.70	13.27[abcd]	0.93	29.89	29.05
Leeudoringstad	nd	3.49	nd	nd	10.89	nd	2.39[a]	4.36	10.30	nd	nd	40.60	6.89	10.35[abcd]	0.83	30.45	29.35
Marquard	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	4.03	25.36	24.39
Ottosdal	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	1.11	31.23	29.92
Potchefstroom	nd	nd	nd	2.90	14.88	nd	2.96[a]	nd	nd	55.60	2.40	26.70	nd	14.12[abcd]	1.89	30.35	29.52
Rushof	14.31	5.24	7.25	nd	nd	nd	4.46[b]	21.00	5.33	58.10	nd	nd	2.19	14.44[abcd]	1.40	30.88	29.78
Tweebuffelsfontein	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	0.56	28.77	28.01
Ventersdorp	nd	nd	18.73	0.84	9.70	17.72	7.83[b]	nd	nd	135.00	1.79	30.00	26.70	32.25[de]	2.63	30.37	29.31
Wesselbron	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	0.28	31.41	30.64
Mean	1.11[a]	1.95[a]	2.43[a]	0.36[a]	2.53[a]	1.32[a]		3.74[a]	15.90[abc]	18.42[abc]	0.38[a]	10.50[abc]	3.10[a]		1.36	29.85	28.77

Different letters shown indicate significant differences (*P*<0.05) between mean fumonisin concentration and mean target DNA in cultivars and at localities based on Fisher's LSD.

ND not detected

Limit of detection =0.016 ppm

Table 2 Mean fumonisin concentration using HPLC analysis, fumonisin-producing target DNA using qPCR, rainfall and maximum temperature during the 2008 maize production season

2008	Fumonisin concentration (ppm)							Target DNA (pg fungal DNA/0.5 g milled maize kernels)							Mean rainfall, Jan, Feb, Mar	Mean max temp, Feb, Mar	Mean max temp, Mar
	DKC 80-12B	DKC 78-15B	PAN 6611	LS8521B	CRN 3505	DKC 80-10	Mean	DKC 80-12B	DKC 78-15B	PAN 6611	LS8521B	CRN 3505	DKC 80-10	Mean			
Bethlehem	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	2.64	24.79	23.30
Bloekomspruit	nd	nd	nd	nd	nd	2.30	0.38[a]	2.47	nd	nd	nd	nd	10.90	2.23[a]	3.29	26.48	25.98
Bothaville	21.84	0.46	12.80	21.84	25.10	19.09	16.85[d]	162.00	6.43	2.93	172.00	6.53	114.00	77.32[bc]	1.83	30.38	29.95
Cedara	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	2.78	25.00	24.25
Coligny	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	2.89	28.50	27.45
Danielsrus	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	na	na	na
Delmas	nd	nd	nd	nd	nd	nd	0.00[a]	nd	2.30	53.00	nd	nd	74.00	21.55[ab]	4.30	26.10	24.70
Frankfort	nd	nd	nd	nd	nd	5.88	0.98[a]	nd	nd	nd	nd	nd	21.30	3.55[a]	2.90	25.98	23.55
Hartebeesfontein	nd	nd	nd	nd	nd	nd	0.00[a]	25.20	nd	nd	nd	3.33	0.21	4.79[a]	3.27	28.71	27.54
Jim Fouche	nd	nd	4.82	nd	nd	3.19	1.34[ab]	nd	nd	15.80	nd	nd	12.60	4.73[a]	1.98	26.04	24.98
Leeudoringstad	nd	nd	4.80	nd	nd	0.94	0.96[a]	13.50	6.72	25.70	4.22	14.00	159.00	37.19[ab]	3.34	28.61	26.92
Nampo	5.67	1.73	nd	nd	26.21	nd	5.60[bc]	117.00	14.20	4.66	nd	569.00	15.00	119.98[c]	2.79	29.76	27.44
Potchefstroom	nd	nd	nd	nd	nd	15.00	2.50[abc]	10.60	3.98	nd	nd	21.30	37.60	12.25[a]	3.37	27.27	25.22
Rushof	nd	nd	nd	nd	6.85	1.58	1.41[ab]	nd	nd	3.24	3.17	6.70	8.63	3.62[a]	29.43	29.25	28.65
Tweebuffels	nd	nd	nd	0.44	nd	nd	0.07[a]	nd	nd	nd	4.67	nd	nd	0.78[a]	14.11	25.34	23.94
Tweeling	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	3.61	27.49	26.54
Vaalharts	nd	nd	5.30	nd	22.53	8.93	6.13[c]	nd	nd	12.90	nd	33.70	96.00	23.77[ab]	1.98	31.41	29.69
Vrede	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	2.29	25.76	25.13
Wonderfontein	nd	nd	nd	nd	nd	nd	0.00[a]	nd	nd	nd	nd	nd	nd	0.00[a]	4.21	26.88	25.49
Mean	1.56[a]	0.27[a]	1.32[a]	1.36[a]	4.13[b]	2.71[a]		16.05[a]	2.26[a]	5.63[a]	9.61[a]	32.40[a]	26.27[a]		5.01	27.43	26.15

Different letters shown indicate significant differences ($P<0.05$) between mean fumonisin concentration and mean target DNA in cultivars and at localities based on Fisher's LSD.

nd not detected, *na* not available

Limit of detection =0.016 ppm

Table 3 Mean fumonisin concentration using HPLC analysis, fumonisin-producing target DNA using qPCR, rainfall and maximum temperature during the 2009 maize production season

2009	DKC 80-12B	DKC 78-15B	PAN 6611	LS8521B	CRN 3505	DKC 80-10	Mean	DKC 80-12B	DKC 78-15B	PAN 6611	LS8521B	CRN 3505	DKC 80-10	Mean	Mean rainfall, Jan, Feb, Mar	Mean max temp, Feb, Mar	Mean max temp, Mar
	Fumonisin concentration (ppm)							Target DNA (pg fungal DNA/0.5 g milled maize kernels)									
Bethlehem	nd	3.18	nd	nd	nd	nd	0.53^{ab}	nd	nd	nd	nd	nd	nd	0.00^{a}	8.95	24.58	24.66
Bloekomspruit	nd	nd	18.88	1.12	nd	2.98	3.83^{bc}	13.50	nd	50.10	18.60	2.76	12.30	16.21^{ab}	3.78	26.67	26.32
Cedara	2.00	nd	nd	nd	nd	1.50	0.58^{ab}	19.40	nd	2.58	nd	nd	2.17	4.03^{a}	2.25	25.86	25.57
Coligny	13.93	nd	nd	nd	nd	nd	2.32^{abc}	7.47	2.27	nd	nd	2.23	nd	2.00^{a}	3.42	30.17	28.78
Delmas	nd	nd	nd	nd	nd	2.50	0.42^{ab}	nd	nd	nd	nd	nd	80.59	13.43^{ab}	3.83	25.78	25.54
Hoogekraal	nd	15.04	nd	nd	6.44	7.00	4.75^{c}	1.74	9.18	nd	7.92	2.71	74.00	15.93^{ab}	3.42	29.50	28.45
Leeudoringstad	1.73	nd	nd	nd	26.71	nd	4.74^{c}	6.87	2.49	6.84	nd	117.00	95.00	38.03^{cd}	0.00	28.76	29.12
Nampo	12.44	14.84	32.68	3.56	19.06	nd	13.76^{e}	17.40	36.70	104.00	13.70	41.40	30.00	40.53^{d}	6.19	29.49	29.72
Ottosdal	1.93	6.35	nd	8.51	4.49	nd	3.55^{bc}	2.23	4.39	1.83	38.50	16.70	21.00	14.11^{ab}	4.88	27.16	27.57
Potchefstroom	nd	0.65	8.11	0.01	8.80	nd	2.93^{abc}	12.11	7.12	3.35	9.21	90.23	16.10	23.02^{bc}	5.91	27.08	26.79
Rushof	0.10	2.66	0.73	0.03	0.03	2.00	0.93^{ab}	nd	33.10	15.10	nd	14.20	12.00	12.40^{ab}	3.44	27.51	26.46
Vaalharts	7.94	3.91	17.41	13.53	9.89	20.92	12.27^{de}	15.75	49.85	40.45	22.55	50.20	28.35	34.53^{cd}	na	na	na
Wesselsbron	4.58	1.89	17.06	2.65	33.26	nd	9.91^{d}	22.90	60.75	22.20	12.40	225.98	nd	57.37^{e}	3.05	28.62	27.99
Wonderfontein	nd	nd	nd	nd	nd	nd	0.00^{a}	nd	nd	nd	nd	nd	nd	0.00^{a}	4.05	27.79	27.74
Mean	3.19^{ab}	3.47^{ab}	6.78^{ab}	2.10^{a}	7.76^{b}	2.64^{ab}		8.53^{a}	14.70^{a}	17.60^{a}	8.78^{a}	40.24^{b}	26.54^{a}		4.09	27.61	27.29

Different letters shown indicate significant differences ($P<0.05$) between mean fumonisin concentration and mean target DNA in cultivars and at localities based on Fisher's LSD.

nd not detected, *na* not available

Limit of detection =0.016 ppm

(February-March). At harvest, kernels from all replications were pooled, thoroughly mixed and a 1 kg sample from each cultivar and locality was collected and stored in a cold-room at 4 °C for a maximum of 1 week prior to milling and further analysis. Sub-samples of 250 g from all the initial 1 kg maize kernel samples were individually milled in a Cyclotech 1093 sample mill with a 1-mm mesh sieve. The mill was thoroughly cleaned with high-pressure air between each sub-sample to minimize cross contamination.

Target DNA quantification

Isolation of fumonisin producing Fusarium spp. DNA for qPCR reactions

A 0.5 g aliquot from each grain sub-sample was used to recover genomic DNA using a DNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions. Prior to qPCR analysis all DNA samples were diluted to 10 ng with Melford molecular grade water using a nanodrop to measure DNA concentrations.

Quantification of fumonisin producing Fusarium spp. using qPCR

MRC 826 (F. verticillioides) strain from the Medical Research Council-Promec Unit was used as the standard in the qPCR technique. This strain is characterized by its ability to produce high concentrations of fumonisins (Gelderblom et al. 2001). To test for linearity and the presence of inhibitors in the pathogen DNA, standard curves were prepared from 10 ng/μl (in triplicate) diluted 10-, 100-, 1000-, 10 000- and 100 000-fold in maize DNA that was free of fungal contamination (10 ng/μl). To assess the presence of inhibitors in maize, the method described by Boutigny et al. (2012) was followed. qPCR reactions were performed in a MyiQ^{TM}2 Two-Colour Real-Time PCR detection system (Bio-Rad, Hercules, USA) with a 96-well reaction plate and Tungsten-halogen optical lamp. The primers Taqfum-2 F and Vpgen-3R in combination with the FUM-probe 1 as tested by Waalwijk et al. (2008) were used in this study. The primer/probe set had the following nucleotide sequence: Taqfum-2 F, 5′-ATGCAAGAGGCGAGGC AA-3′; Vpgen-3R, 5′-GGCTCTCRGAGCTTGGCAT-3′ and FUM-probe 1,5′-/56-FAM/CAATGCCATCTT CTTG/36-TAMSp/-3′. The sensimix reagent kit (sensimix^{TM}no ref QT 505–05) from Celtic (Bioline)

was used for qPCR. For each reaction, 4 μl of the DNA sample was mixed with 12.5 μl sensimix, 2.125 μl Fum-probe 1 (1 μM), 0.875 μl Taqfum-2 F (333 nM), 0.875 μl Vpgen-3R (333 nM) and 4.625 μl Melford molecular grade water. No template controls were subjected to the same procedure to exclude or detect any possible contamination or carryover. The 96-well plate was incubated for 10 min at 95 °C and thereafter, each of the 40 PCR cycles was performed according to the following temperature regime: 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 10 s. The Bio-Rad iCycler^{Tm}iQ Optical System Software Version 3.0a was used to calculate the concentrations of target DNA of Fusarium spp. that produce fumonisins (picogram fungal DNA/ 0.5 g ground maize kernels) present in a sample. Standard curves from runs yielded a highly significant relationship ($R^2 \geq 0.99$). Slopes were within the acceptance criterion (between −3.1 and −3.6) and efficiencies ranged from 95 to 105 %.

Mycotoxin analyses

Mycotoxins were analysed using the Vicam method (Anonymous 2002). A 50 g aliquot from each of the 250 g milled sub-samples was mixed with 5 g of sodium chloride prior to extraction. Fumonisin were extracted in 100 ml methanol:water (80:20, v/v) for five min using an IKA T18 basic Ultra Turrax homogeniser. The extract was then filtered through Whatman No.5 filter paper. A 10 ml aliquot was diluted with 40 ml phosphate-buffered saline (1XPBS) (8.0 g NaCI, 1.2 g Na2HPO4, 0.2 g KH2PO4, 0.2 g KCI, dissolved in 990 ml purified water with pH adjusted to 7.0 with HCI). Diluted samples were extracted through microfibre filters (0.45 μm) and 10 ml of the diluted filtrate was passed through FumoniTest affinity columns from Vicam at a flow rate of 1 drop per second. Ten ml of PBS were subsequently passed through the column at a rate of 1 drop per second. The column was washed with 1.5 ml HPLC grade methanol at a rate of 1 drop per second and the eluate was collected in a glass cuvette. Methanol eluate was dried in a TurboVap LV (Caliper Sciences) with the aid of a slow stream of high purity Nitrogen. Samples were dissolved in 200 μl methanol and purified water (50:50 v/v). Each sample (50 μl) was transferred to 250 μl conical inserts. Each insert was placed into a 2.5 ml glass vial which was then placed into a carousel. The first position of the carousel had a 2.5 ml glass vial with o-phthaldialdehyde (OPA from

Sigma) which is the derivatisation agent. The Waters 717 plus autosampler was set up to mix 100 μl of the OPA with the 50 μl of sample in the conical insert. This mixture (20 μl) was injected after a delay time of 1 min.

Fluorescence was performed at excitation and emission wavelengths of 335 and 440 nm respectively using a Waters 2475 multi λ fluorescence detector. The analytical column, Symmetry C18, 5 μm 3.9×150 mm from Waters was used. The detection limit of the method used was 0.016 ppm. Recovery data were obtained in triplicate by fortifying uncontaminated maize samples (Vicam) with 5 ppm fumonisin B_1 B_2 and B_3. The average recovery rates were 83 % (FB_1), 81 % (FB_2) and 83 % (FB_3).

Isolation, identification and quantification of Fusarium spp. using the plating out method

The quantification of *Fusarium* spp. using the plating-out method was done using 200 surface-sterilised (King and Scott 1981) kernels from each of the maize samples from the 2007 season. Kernels were plated onto Rose Bengal-glycerine-urea agar (van Wyk et al. 1986). Four kernels were plated equidistantly on a petri dish (Flett 1994) and petri dishes were incubated for 7 days in the dark at 25 °C (Nelson et al. 1983; Leslie and Summerell 2006). After 7 days, fungal colonies were transferred to carnation leaf agar (CLA)/potato dextrose agar (PDA) for identification. The broad spectrum antibiotic chloramphenicol was applied at a rate of 250 mg per 1 l agar to both CLA and PDA to inhibit bacterial growth (Leslie and Summerell 2006). CLA/PDA cultures were placed under ultraviolet light to promote the development of spore chains and the teleomorph stage to aid identification (Leslie and Summerell 2006). Ultraviolet lighting was programmed to provide 12 h light and 12 h darkness. After 14 days, *Fusarium* spp. were identified using the guidelines of Nelson et al. (1983) and Leslie and Summerell (2006).

Data analysis

Incidence of fumonisin producing Fusarium spp. and fumonisins

Target DNA and fumonisin concentrations were analyzed using two factor analysis of variance without replication to determine the effect of locality and season on grain colonization and fumonisin production.

GXE interactions

Spearman Rank Correlations were used to determine the consistency of ranking of reactions of cultivars to fungal and fumonisin contamination over localities and seasons. Non-linear regression analysis ($Y=aX^b$) was used to determine the relationship between *Fusarium* colonization/fumonisin contamination potential at each locality and the observed response of each cultivar (Flett and McLaren 1994). *Fusarium* colonization / fumonisin contamination potential of a locality was defined as the mean *Fusarium* colonization / fumonisin contamination level over all cultivars at that locality and was regarded as in index which reflects causal conditions at that locality. The generated graphs and model parameters reflect the response of the test genotype to changing potentials and thus reflect the stability of the response to changing conditions. In the model $Y=aX^b$, Y=mean fungal colonization/fumonisin contamination within each genotype and X=fungal colonization/fumonisin potential of the respective localities. Three types of relationships were anticipated based on the results of McLaren (1992) and Flett and McLaren (1994). These relationships are defined by the *b* parameter. Where $b=\pm1$, a linear relationship between colonization/fumonisin potential and observed colonization/fumonisin exist, where in $b\geq1$, various degrees of resistance despite an increasing disease / fumonisin potential exist and $b\leq1$, susceptibility to disease / fumonisin existed despite a low disease / fumonisin potential.

Correlation between qPCR, HPLC, and the plating out method

Simple linear regression analysis using Statgraphics version 5.0 was performed to determine the relationship between the plating-out method, quantification of fumonisin-producing *Fusarium* spp. using qPCR and fumonisins.

Relationship between maximum temperature and rainfall, grain colonization and fumonisin production

Simple linear regression analysis (Statgraphics version 5.0) was performed to determine the relationship

between mean maximum temperature and maximum rainfall during January to March of each year and fumonisin concentration and mean fumonisin producing *Fusarium* spp. DNA.

Results

Target DNA and fumonisin concentration over seasons, localities and cultivars

Fumonisin producing *Fusarium* spp. (Tables 1, 2, and 3) and fumonisin concentrations (Fig. 1) were quantified from maize samples from North West, Gauteng, Free State, Mpumalanga, KwaZulu- Natal and Northern Cape provinces from 2007 to 2009. One way analysis of variance indicated highly significant locality effects on target DNA (Tables 1, 2, and 3). The highest target DNA of these fungi in 2007 were recorded in Rushof (48.77 pg) and Ventersdorp (38.65 pg) while no fumonisin-producing *Fusarium* spp. were recorded in Bethlehem, Marquard and Ottosdal (Table 1). During the 2008 season, high amount of target DNA were recorded in Nampo (119.98 pg) and Bothaville (77.32 pg) with target DNA recorded at seven localities (Table 2). In the 2009 season, high amounts of target DNA were recorded at Wesselsbron (70.87 pg) and Nampo (42.64 pg) with no target DNA being recorded at Bethlehem, Delmas and Wonderfontein (Table 3).

One way analysis of variance indicated highly significant locality effects on fumonisin concentrations (Tables 1, 2 and 3). In 2007 highest fumonisin concentrations were recorded at Ventersdorp (7.83 ppm) and Rushof (4.46 ppm), in 2008 at Bothaville (16.85 ppm), Vaalharts (6.13 ppm) and Nampo (5.60 ppm), and in 2009 Nampo (16.52 ppm) and Wesselsbron (14.39 ppm). No fumonisins were recorded from

various locations during 2007–2008 and Delmas and Wonderfontein in 2009 (Tables 1, 2, and 3).

Although a highly significant relationship was recorded between target DNA and mean fumonisin concentrations (R^2=0.63, P=0.000, 42df; Fig. 2), high variation in both target DNA (0–225 pg) and fumonisin concentration (0–33.26 ppm) were recorded with high seasonal x locality interactions. During 2007 for example, a high amount of target DNA of 21.3 pg in DKC80-12B at Hartebeesfontein, resulted in no fumonisin production while at Koster low target DNA levels (2.11 pg in DKC80-12B) were associated with high fumonisin concentrations (15.24 ppm). Similar deviations were observed during the 2008 and 2009 seasons.

Analysis of variance indicated significant differences in target DNA due to cultivar in the 2007 and 2009 seasons but not the 2008 season. In the 2007 season, highest mean amount of target DNA was recorded in cultivar PAN6611 (18.42 pg) with the lowest target DNA in LS8521B (0.38 pg). During 2009, the highest target DNA was recorded in CRN3505 (43.34 pg) and the lowest in DKC80-12B (8.14 pg). Spearman Rank Correlations indicated that the responses of cultivars to colonization of grain by fumonisin producing *Fusarium* spp. varied over localities within seasons (r_s=0.42 to 0.64) suggesting that cultivars reacted differently to different environmental/inoculum conditions (disease potentials). The application of non-linear regression analyses in an attempt to quantify the relationship between observed cultivar response and colonization potential yielded limited success (Fig. 3a–f). Based on regression parameters (a<1), cultivars CRN3505 and DKC80–12B showed a degree of resistance to colonization despite increasing colonization potential while poor relationships were recorded with remaining cultivars.
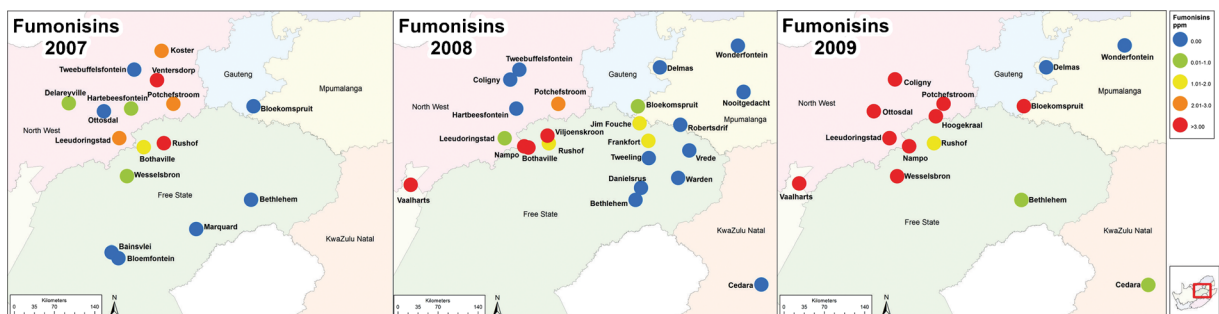
Fig. 1 Fumonisin levels quantified in the commercial maize production areas of North West, Gauteng, Free State, Mpumalanga, KwaZulu-Natal and Northern Cape provinces during 2007–2009 using HPLC
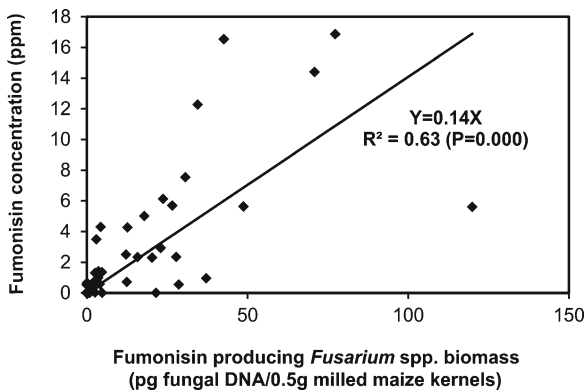
Fig. 2 Simple linear regression analysis of the relationship between mean fumonisin-producing *Fusarium* spp. target DNA determined using qPCR and mean fumonisin concentrations using HPLC

Analysis of variance indicated no significant differences in mean fumonisin concentrations due to cultivar in the 2007 and 2009 seasons (Tables 1, 2 and 3). In the 2008 season, CRN3505 had a significantly higher mean fumonisin concentration compared to the rest of the cultivars. As with fungal colonization, Spearman Rank Correlations indicated the response of cultivars to fumonisin contamination to be poorly correlated over localities/seasons (r_s=0.29 to 0.70). Non-linear regression analyses of the relationship between observed cultivar response and fumonisin potential proved more successful (Fig. 3g–l). Observed fumonisin concentrations in cultivars CRN3505 and PAN6611 were linearly related to fumonisin potential (a=1), while DKC80-12B and LS8521B showed a degree of resistance despite increasing fumonisin contamination potential (a<1), DKC78-15B was susceptible (a>1) and cultivar DKC80-10 yielded a non-significant response to fumonisin potential.

Correlation between qPCR, HPLC, and the plating out method

A simple regression analysis between the plating out method and the qPCR method yielded no significant relationship (R^2=0.04; P=0.429). A highly significant relationship was recorded between mean target DNA using qPCR and mean fumonisin concentrations with a R^2=0.63, P=0.000. This corresponds with the findings of Ncube et al. (2011) who reported a poor correlation between *F. verticillioides* quantified by the plating out method and target DNA of *Fusarium* spp. determined

by qPCR (r=0.14, $p \leq 0.05$). This suggest qPCR to be a better indicator of potential fumonisin contamination.

Relationship between maximum temperature and rainfall, grain colonization and fumonisin production

The monthly mean maximum temperature and rainfall from January to March (2007–2009) for each locality were calculated (Tables 1, 2 and 3) to determine relationships between weather and infection of kernels by *Fusarium* spp.. No significant relationship between these weather parameters and colonization of grain by fumonisin producing *Fusarium* spp. was recorded, although a tendency was observed for higher fungal infection at warmer localities while low or zero infection was associated with cooler areas.

Mean maximum temperature during February and March for the 2008 season showed a significant relationship with fumonisin contamination (R^2=0.64, P=0.047, 12 df; Fig. 4). Similarly, a significant relationship between mean maximum temperature for March and fumonisin concentration was recorded during the 2009 season (R^2=0.67; P=0.042, 12 df) while no relationship was recorded during 2007. No significant relationship was recorded between mean rainfall and fumonisin concentration.

Discussion

F. verticillioides and *F. proliferatum* are the most prolific fumonisin-producing *Fusarium* spp. (Shephard et al. 1996) and are generally associated with warm, dry climates (Shephard et al. 1996; Fandohan et al. 2003; Munkvold 2003). The natural occurrence of fumonisin-producing *Fusarium* spp. in maize samples from 29 localities over a 3-year period indicates that infection is common in South African commercial maize samples, especially in the warmer, drier areas such as the North West province, Northern Cape and some areas of the Free State such as Bothaville, Nampo, Rushof and Wesselsbron, where the average temperatures ranged from 29 °C to 32 °C. In cooler areas such as Bethlehem, Bloekomspruit, Cedara, Danielsrus, Delmas, Tweeling, Vrede, Warden and Wonderfontein, where mean maximum temperatures ranged from 24 °C to 27 °C, target DNA levels were low or not detected. Murillo-Williams and Munkvold (2009) calculated
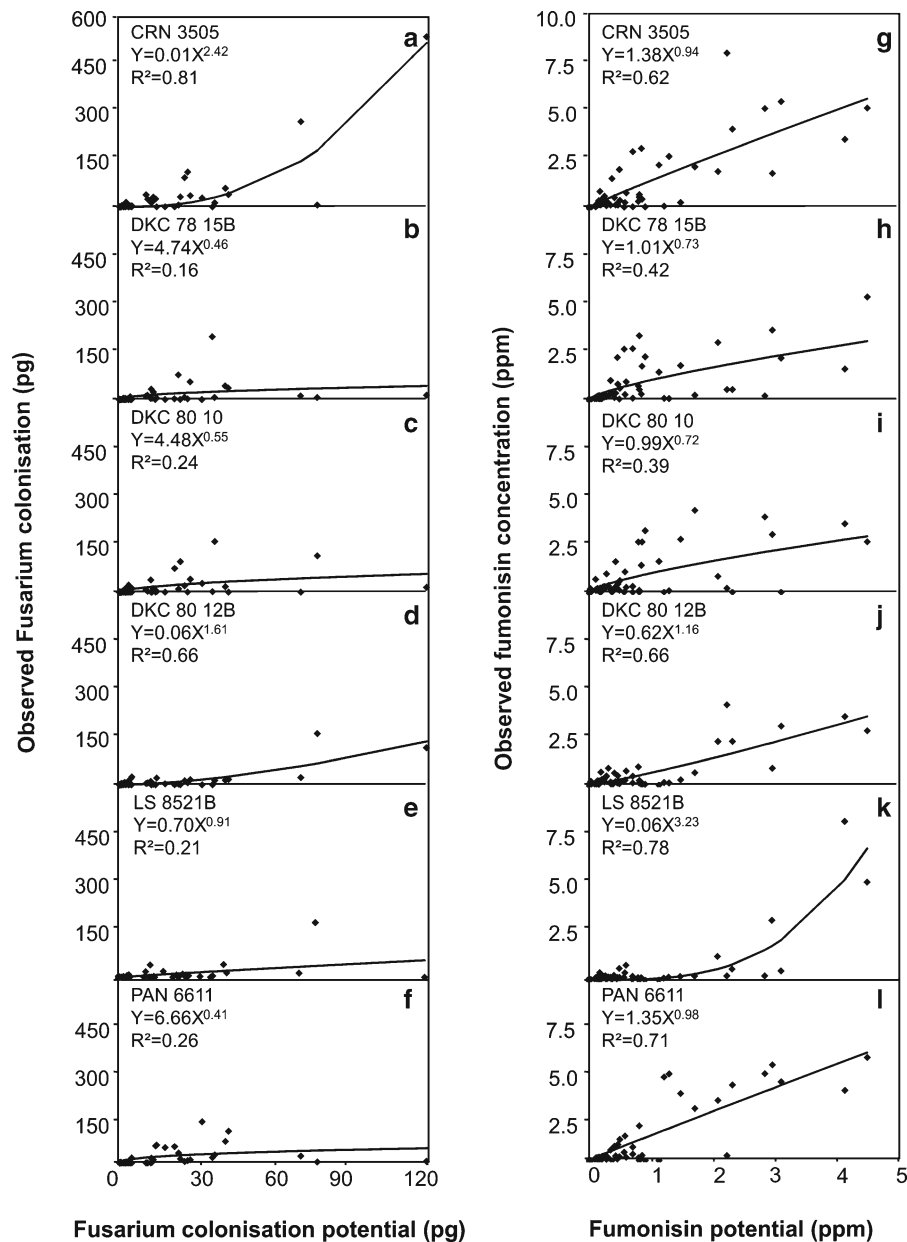
Fig. 3 Non-linear regression analysis ($Y=aX^b$) of the relationship between the responses of six maize genotypes to colonization by fumonisin-producing *Fusarium* spp. as indicated by target DNA measured with qPCR and fumonisin contamination determined using HPLC with changing potentials

optimum and supra-maximal temperatures for the growth of *F. verticillioides* at 31 °C and 35 °C respectively, with minimal fungal growth at 22 °C to 24 °C. Similarly in this study, highest fumonisin ($FB_1+FB_2+FB_3$) concentrations were detected in warmer areas such as Ventersdorp, Rushof, Bothaville, Vaalharts, Nampo, and Wesselsbron with low to undetectable fumonisin concentrations in the cooler areas and this is consistent with Munkvold (2003) and De La Campa et al. (2005) who reported optimum temperatures of 30 °C and 32 °C for fumonisin production.

At present, the only two mycotoxins considered under South African national regulations are aflatoxin (in all foodstuffs) and patulin (apple juice) (Rheeder 2008). No legislation on allowable concentrations for Fusarium mycotoxins in South Africa has been formulated
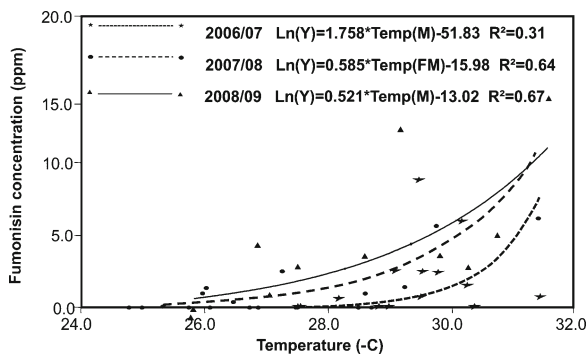
Fig. 4 Simple linear regression analysis to determine the relationship between mean maximum temperature and maximum rainfall during January to March of each year and fumonisin concentration determined using HPLC and mean fumonisin-producing *Fusarium* spp. target DNA using qPCR

(Fandohan et al. 2003). The European Union established a limit of 1 ppm (FB_1) for food intended for direct human consumption, whereas the United States Food and Drug Administration is currently suggesting a guideline of 2 ppm (FB_1) for direct human consumption when considering food safety in local maize products. High fumonisin concentrations in excess of 2 ppm were recorded at 10 localities over the 3-year period. These concentrations are of concern because of possible mycotoxicoses in animals (Thiel et al. 1991) and carcinogenic effects in humans as described by the World Health Organizations International Agency for Research on Cancer (WHO-IARC, 1993) as well as Marasas et al. (1981, 1988) and Rheeder et al. (1992).

In the absence of South African fumonisin regulations, no official testing for fumonisin contamination of grain is conducted in South Africa to prevent high levels of contamination, as recorded at some localities, entering the food chain. Internationally, analysis of mycotoxins relies heavily on HPLC (Pascale and Visconti 2008) as this method has the advantage of good sensitivity, selectivity and repeatability. Due to expensive equipment which requires specialist expertise, as some toxins may need derivitisation, a paucity of equipment and expertise exists in South Africa to screen maize samples at all delivery points. South African grading regulations, for commercial grain at silos aims to reduce the risk of visually contaminated maize being consumed by humans and animals based on observed discoloration and physical defects and resultant downgrading of grain. However, endophytic infections in maize are difficult to detect because kernels appear sound (symptomless) and escape detection, this may result in fumonisins entering

the food chain. This study showed that although a highly significant relationship ($R^2=0.63$; $P=0.000$) between target DNA and fumonisin concentration were recorded, deviations occurred. High target DNA with low fumonisin content and low target DNA with high fumonisin content were recorded at certain localities/seasons/cultivars. Similarly Bush et al. (2003) reported that infection in symptomless kernels was often very high with low fumonisin concentrations. In contrast Bacon et al. (2008) reported that symptomless infections can produce high concentrations of fumonisin.

Surveys of the natural occurrence of fumonisin producing *Fusarium* spp. and fumonisins in commercial maize production areas of South Africa are necessary to assess the potential threat of these toxins to humans and animals especially in view of the constantly changing cultivars that enter the market. It is therefore important that the level of infection and fumonisin risk be accurately determined to ensure safe food for human and animal consumption. Corresponding high target DNA values may also indicate infection levels that may reduce yields and cause grain discolouration, physical breakdown of grain structure and reduction of grain nutritional value (Jurado et al. 2006) although these were not quantified in the current study.

Results recorded in the present study indicate that maize cultivars differ in susceptibility to colonization by fumonisin-producing *Fusarium* spp. Cultivars CRN3505 and DKC80-12B showed a degree of resistance to fungal infection. However the economic value of the differences are limited due to the high genotype x environment interactions recorded (2007 and 2009) and indicates the need for studies under local conditions on the stability of host response to different isolates or changing environmental conditions. Similar genotype x environment interactions were also reported for Fusarium ear rot and fumonisins by Small et al. (2012). Inconsistent responses of cultivars over environments could be due to variation in isolates, inoculum levels and adaptation of genotypes to physiological stress factors (Munkvold 2003; Desjardins 2006). Host predisposition by physiological stresses and disease-favourable conditions prior to harvesting may create conditions for infection by *Fusarium* spp. and fumonisin production (Visconti 1996). It is also plausible that general agronomic practices such as soil fertility, irrigation and cropping systems could have affected the observed responses. These results

illustrate the importance of taking the G x E inter-action into account when evaluating cultivars for resistance to Fusarium ear rot of maize as well as the need to define evaluation criterion in breeding for Fusarium resistance. The absence of a significant relationship between potential and observed cultivar response in most cultivars would suggest a differential response in genotypes to potential as opposed to a uniform response to changing potential as reflected in CRN3505 and DCK80-12B and the *Claviseps africana*, *Stenocarpella maydis* and *Sclerotinia sclerotiorum* pathosystems used by McLaren (1992), Flett and McLaren (1994) and McLaren and Craven (2008), respectively. This suggest that numerous effects of locality on both the pathogen and host still need to be elucidated.

The absence of a relationship in this study between the plating-out method and quantification of fumonisin-producing *Fusarium* spp. using qPCR may be due to the plating-out method reflecting the number of viable spores or mycelia that will germinate on agar as opposed to the actual fungal biomass of grains. Mycelial fragments will give rise to only one colony, even if they consist of many cells (Passone et al. 2010). In a qPCR reaction, viable and dead cells can contribute to the number of *fum1* copies and as a result give a more accurate indication of actual target DNA present. The correlation between qPCR and fumonisin concentration using HPLC was significant, although variation in R^2-values in regression analyses from one season to another may be due to the fumonisin biosynthetic gene, *fum1* being present in the sample, but not always expressed. The qPCR method used in this study will quantify the biomass of fumonisin-producing *Fusarium* spp. (pathogenic and/or endophytic) more accurately, thereby giving a better understanding of the potential risk of grain being contaminated with fumonisin. It is not foreseen that this method will be applied at silos because of the expertise, time and cost factors involved, but it can be applied successfully in other applications ensuring food safety and quality to humans and animals.

A better understanding of the role of environment and the physiology of differential response of cultivars in relation to infection of fumonisin-producing *Fusarium* spp. at different localities is essential to the identification of maize production areas with a potential high/low risk of fumonisin contamination.

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