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Pathogenicity and toxigenicity of *Fusarium verticillioides* isolates collected from maize roots, stems and ears in South Africa

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Abstract *Fusarium verticillioides* is most frequently associated with maize in South Africa. It colonises maize roots, stems and ears endophytically and causes diseases such as Fusarium ear rot (FER) and stalk rot. *Fusarium verticillioides* can produce fumonisins, which are toxic secondary metabolites harmful to humans and animals. It is, however, unknown whether endophytic and pathogenic isolates from distinct maize tissues differ in their ability to cause disease and produce fumonisins. In this study, *Fusarium* spp. were collected from maize roots, stems and kernels for phylogenetic analysis and the *F. verticillioides* isolates were subjected to pathogenic and toxigenic comparison. The translation elongation 1-α (TEF1) gene of the isolates was sequenced, and a phylogenetic tree constructed with maximum likelihood (ML) and Bayesian interference (BI) inferred. Fumonisin production of *F. verticillioides* isolates was determined *in vitro* and *in planta* by using high performance liquid chromatography, and virulence of the isolates was determined by silk channel inoculation of maize ears under field conditions. *F. verticillioides* was the species with the highest number of isolates followed by *F. temperatum* and then *F. subglutinans*. Phylogenetic analyses clustered the different *Fusarium* spp. according to species. Fumonisin production by *F. verticillioides* isolates varied from 0 to 21.3 mg/kg *in vitro*, and 0–16.2 mg/kg *in planta*. All the *F. verticillioides* isolates produced FER symptoms, including isolates from roots and stems. *Fusarium verticillioides* isolates in South Africa thus presented a species highly diverse in toxigenicity, but not in virulence. This finding has implications for managing mycotoxins in maize, as visible symptoms might be misleading to the actual toxin level present, for example low level of disease severity might represent high fumonisin levels and *vice versa*. The high numbers of *F. temperatum*, also a mycotoxin producer highlights the concern that kernels could be contaminated with more than one mycotoxin. Integrated disease management of not only *F. verticillioides* but all *Fusarium* spp. should thus focus strongly on reducing fungal contamination of maize and the detoxification of grain with focus on using regionally adapted maize varieties.

Keywords Fumonisins · *Fusarium verticillioides* · Pathogenicity · Phylogeny · Toxigenicity

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Introduction

Fusarium verticillioides (Saccardo) Nirenberg is a fungus associated with many plants such as teosinte (*Zea* spp. (Schrad) Kuntze), millet (*Pennisetum glaucum* (Linnaeus) Brown), sorghum (*Sorghum bicolor* (L.) Moench) and tallgrass (Talasium Spreng). It can also

be pathogenic to humans by infecting the cornea, thereby causing keratitis (Desjardins et al. 2000; Hirata et al. 2001; Leslie et al. 2004, 2005; O'Donnell et al. 2007). *Fusarium verticillioides*, however, is best known for infecting maize plants wherever the crop is cultivated (Nelson et al. 1983; Leslie et al. 1990; Leslie 1991; Shephard et al. 1996). It colonizes plant roots, stems and seed as an endophyte (Bacon and Hinton 1996; Munkvold and Desjardins 1997), and can frequently be isolated from asymptomatic tissue (Foley 1962; Kedera et al. 1994; Bacon et al. 2001). In maize ears, *F. verticillioides* can cause Fusarium ear rot (FER), a disease that affects the quality and quantity of healthy maize grain. Other *Fusarium* spp. also part of the FER complex in fewer numbers are *F. proliferatum* (Matsushima) Nirenberg and *F. subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas (Nelson et al. 1983; Leslie et al. 1990).

A major characteristic of *F. verticillioides* is its ability to produce mycotoxins, of which the fumonisins are the most important. High levels of fumonisins have a negative effect on consumers of maize grain, since it can be carcinogenic to humans and animals (Gelderblom et al. 1991; Marasas 1996). Increased levels of fumonisins in mouldy maize kernels has been statistically linked to human oesophageal cancer in the former Transkei region in South Africa (Rheeder et al. 1992), China (Chu and Li 1994), Polenta in northern Italy (Pascale et al. 1995) and in the Santa Catarina State in southern Brazil (Van der Westhuizen et al. 2003). The mycotoxin has also been associated with neural tube defects in humans in the former Transkei region in South Africa, in northern China and Mexico (Marasas et al. 2004; Missmer et al. 2006). In animals it causes equine leukoencephalomalacia in horses (Kellerman et al. 1990), pulmonary oedema in pigs (Harrison et al. 1990) and hepatocarcinogenesis in rats (Gelderblom et al. 1991).

In South Africa, fumonisin levels of more than 2 mg/kg were recorded in commercial cultivars in the North West and Free State provinces, while only trace amounts were measured in commercial maize grown in Gauteng, Mpumalanga and KwaZulu-Natal (Janse van Rensburg 2012). An amount of 2 mg/kg is the maximum tolerable daily intake recommended by the Joint FAO/WHO Expert Committee on Food Additives (World Health Organization 2002). The South African Ministry of Health has in September 2016 implemented new regulations that allow 2 and 4 mg/kg fumonisin for maize flour/meal for human consumption and raw maize, respectively (www.gpwonline.co.za). In rural maize production areas of South Africa, however, much higher fumonisin levels are recorded. Rheeder et al. (1992) and Ncube et al. (2011) have reported fumonisin levels of up to 21.8 mg/kg in Mokopane and Venda (Limpopo Province), Lusikisiki (Eastern Cape Province), Mbazwane, Jozini, Pongola and Manguzi (Northern KwaZulu-Natal Province), whereas FB_1 levels of up to 117 mg/kg were found in the Centane and Butterworth districts in the former Transkei (Eastern Cape Province). In Centane, fumonisin levels recorded in home-grown maize were also much higher (1142 ppb) than that found in commercial maize (222 ppb) (Burger et al. 2010). The difference in fumonisin levels found in maize grown under commercial and resource-poor production systems might be the result of resource-poor farmers planting home-grown seed from the previous season as well as not applying recommended management practices. Alternatively, *F. verticillioides* isolates found in different fields might contribute to the variation in fumonisin levels.

Fusarium verticillioides isolates that differ in the amount of fumonisin they produce have been reported (Marasas 1996), with some isolates producing little or no fumonisin at all (Desjardins et al. 1995; Desjardins and Plattner 2000). Different amounts of fumonisins are also produced by isolates collected in different regions. For instance, *F. verticillioides* isolates collected in northern Luzon in the Phillipines produced more fumonisins than those in the southern part of Luzon (Cumagun et al. 2009). These differences in the toxigenic potential of *F. verticillioides* isolates could be due to mutations in the fumonisin (FUM) biosynthetic gene cluster, which renders one or more genes non-functional (Proctor et al. 2004). If FUM1, FUM6 and FUM8 genes are disrupted due to a mutation, fumonisin production is completely terminated (Seo et al. 2001). Fumonisin production, however, is not required for FER to be inflicted by *F. verticillioides*. For instance, Desjardins et al. (1995) demonstrated that a non-fumonisin-producing field strain of *F. verticillioides* with a mutation in the FUM1 gene was still pathogenic to maize. Jardine and Leslie (1999) also found that stalk lesion size and *in vitro* fumonisin production did not correlate, and that different *F. verticillioides* strains produced different levels of fumonisins.

The identification of *F. verticillioides* can sometimes be difficult. The species is morphologically similar to

F. thapsinum Klittich, Leslie, Nelson and Marasas (Klittich et al. 1997), *F. fujikuroi* Nirenberg (Leslie and Summerell 2006), *F. musae* Van Hove, Waalwijk, Logrieco, Munaut and Moretti (Van Hove et al. 2011), *F. proliferatum*, *F. andiyazi* Marasas, Rheeder, Lamprecht, Zeller and Leslie (Marasas et al. 2001) and *F. nygamai* Burgess and Trimboli (Burgess and Trimboli 1986; Klaasen and Nelson 1996; Nirenberg and O'Donnell 1998; Leslie and Summerell 2006). It has been named *F. moniliforme* before, a species that included *F. thapsinum*, *F. saccahari* (E.J. Butler) W. Gams (Butler and Khan 1913), *F. mangiferae* Britz, Wingfield and Marasas (Britz et al. 2002) and *F. fujikuroi* Nirenberg (Leslie and Summerell 2006). Molecular techniques, such as sequencing of the translation elongation factor 1-α (TEF1) gene is used to overcome the limitations of morphological species identification. Genotypic data from sequencing can further be used to separate *Fusarium* species into phylogenetic species or lineages (O'Donnell et al. 1998, 2000). Phenotypic information, such as mycotoxin production and plant host specificity, has also been used before to separate closely-related *Fusarium* species (Scauflaire et al. 2011a; Van Hove et al. 2011).

In this study, *F. verticillioides* and related *Fusarium* spp. isolates from maize roots, stems and ears collected in six South African provinces were identified at the molecular level. The toxigenic potential and virulence of *F. verticillioides* were also determined to improve our understanding of FER and fumonisin contamination of maize in different production regions of the country and isolated from different maize tissues. Phylogenetic analysis was used to distinguish between *F. verticillioides* and closely related *Fusarium* species.

Materials and methods

Isolates used

Fusarium spp. were isolated from maize kernels collected in the Eastern Cape (13 localities), Limpopo (seven localities), Mpumalanga (13 localities), Gauteng (one locality) and KwaZulu-Natal (17 localities); and from maize kernels, stems and roots collected in the Free State (ten localities) and North-West (eight localities) provinces of South Africa during seasons 2007/08. Forty kernels from each maize plant were surface disinfected for 5 min in 2% NaClO and washed three times with sterile water, four maize kernels were placed on each of ten Petri dishes containing Van Wyk's medium (Van Wyk et al. 1986). The roots and stems (from roots until the first node) were cut into 1-cm pieces, surface sterilized as described above, and plated onto Van Wyk's medium (Van Wyk et al. 1986). Sixty root and stem pieces from each plant were plated out, with four pieces per plate. These plates were then incubated at 25 °C under cool-white and near-ultraviolet fluorescent lights. Developing colonies tentatively identified as *Fusarium* spp. were transferred to potato dextrose agar (PDA) (39 g Difco, 1000 ml H_2O), and incubated for 7 days at conditions described above. They were then single-spored (Nelson et al. 1983), preserved in 15% glycerol and maintained at the culture collection at the Agricultural Research Council-Grain Crops Institute, Potchefstroom. *Fusarium verticillioides* isolate MRC 826, a prolific producer of fumonisin (Rheeder et al. 2002), was included as control isolate.

DNA extraction

DNA was extracted from all the *Fusarium* isolates collected from maize kernels, roots and stems. Cultures were first grown on PDA plates for 7 days at 25 °C, and DNA extracted by using the method described by Sambrooks et al. (1989). It was visualised on 1% agarose gels (w/v), stained with Gel Red (Biotium Inc., Hayward, CA), and viewed using the Geldoc system (Bio-rad, Hercules, USA). A molecular marker (hyperladder I) (Bioline, London, UK) was used to determine the size of the DNA. The concentration of the DNA was measured with a nanodrop (NanoDrop, Wilmington, USA) and adjusted to 20 ng/μl. The DNA was kept at −20 °C until analysis.

Identification of *Fusarium* isolates

Morphological identification Each single-spore isolate of *Fusarium* was plated onto carnation leaf agar (CLA) (20 g of Biolab agar, 1000 ml H_2O, one or two sterilized carnation leaves) and PDA for morphological and cultural identification, respectively. All plates were incubated at 25 °C as described above. After 7 days of growth, isolates were morphologically identified (Nelson et al. 1983) in order to select

F. verticillioides and all other morphologically-related *Fusarium* isolates.

Molecular identification The PCR products were visualized. *Fusarium* isolates (415) were identified by sequencing of the translation elongation factor 1-α (TEF1) gene. The TEF1 region was amplified with the forward primer EF1 (5′ ATG GGT AAG GAG GAC AAG AC 3′) and the reverse primer EF2 (5′ GGA GGT ACC AGT GAT CAT GTT 3′), using PCR conditions as described by O'Donnell et al. (1998). A negative control (water) was included in each reaction, as well as DNA of a known *F. verticillioides* isolate that served as positive control. The PCR products were visualized on 1% agarose gels, as described above. A 100-bp molecular weight marker Generuler™ 100 bp (Fermentas, Hanover, USA) was used to determine the size of the PCR products.

The PCR product was cleaned up for sequencing using the Zymoclean kit (Zymo Research Corporation, Irvine, CA, USA). Sequencing reactions were performed in a 3100 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator v3.1. kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequenced data of the isolates were aligned using Chromas and Bioedit v7.0.9.0 software (www.mbio.ncsu.edu/BioEdit/BioEdit.html). The aligned sequences were then submitted to the basic local alignment search tool (BLAST) against the Fusarium ID database (Geiser et al. 2004; O'Donnell et al. 2010) and the NCBI (http://www.ncbi.nlm.nih.gov/) in order to confirm the identity of the isolates.

Phylogenetic analysis A phylogenetic tree was constructed for the TEF1 gene data set containing the *Fusarium* spp. isolated from maize kernels, roots and stems. Phylogeny based on Bayesian interference (BI) and maximum likelihood (ML) methods were inferred for the data set using Mr. Bayes version 3.1.2 (Heulsenbeck et al. 2001), and PhyML, version 3.0 (Guidon and Gascuel 2003), respectively. For these analyses the best-fit model of evolution (TPM3 + G) (Posada 2003), as indicated by the jmodeltest 0.1 package (Posada 2008), was used. BI trees were constructed using the Metropolis-coupled Monte Carlo Markov chain with 2 million generations, after which Bayesian posterior probabilities were calculated. ML bootstrap confidence values were based on 1000 replications using the parameters of the TPM3 + G evolution model.

Fumonisin production by *Fusarium verticillioides* isolates

In vitro fumonisin production: Fumonisin production of 291 *F. verticillioides* isolates collected from maize in South Africa was determined *in vitro*. Isolates were cultured first in Armstrong media for 4 days to obtain conidial suspensions of 1×10^8 conidia ml^{-1} for each isolate. An aliquot of 500 μl spore suspension per isolate was then inoculated into 250-ml Erlenmeyer flasks filled with 50 ml fumonisin-producing medium to give a final spore concentration of 110^6 conidia.ml^{-1} (Jiménez et al. 2003). The flasks were incubated at 20 °C for 4 weeks in static conditions, after which the liquid media were filtered through Whatman no.1 paper (Merck Millipore). The media were again filtered through a syringe nitrocellulose filter with a pore size of 0.22 μm (Merck Millipore), and the filtrate stored at −20 °C for a week until fumonisins could be measured.

In planta fumonisin production: Fifteen *F. verticillioides* isolates differing in *in vitro* fumonisin production were selected for field evaluation. The *F. verticillioides* isolates selected included isolates that produced more than 2 mg/kg, less than 2 mg/kg, or no fumonisins. The primary ears of maize plants were inoculated through the silk channel with 2-ml aliquots of a 1×10^6 conidia.ml^{-1} spore suspension 1 week after silking (Afolabi et al. 2007). Control plants were inoculated with water. The experimental set-up was a randomised complete block design, and the experiment was replicated three times. At harvest, inoculated ears were handpicked at 12% kernel moisture level, threshed, and a 250-g maize kernel sample milled to pass through a 1-mm mesh using a Cyclotech sample mill (Foss Tecator, Hoganas, Sweden). From the milled maize, a 50-g sample was used for fumonsin extraction (which was also stored at −20 °C for no longer than a week) using the Fumonitest™ HPLC method from VICAM (Watertown, USA) according to the manufacturer's instructions.

Fumonisin analysis: Fumonisin analyses of the *F. verticillioides* culture filtrates and maize samples were performed by HPLC using a reverse-phase HPLC/fluorescence detector system (Waters, Massachusetts, USA). Two ml of each filtrate was prepared for testing fumonisin production *in vitro* as described by López-Errasquín et al. (2007). In this method, the fumonisins were first eluted with 14 ml of 1% acetic acid in methanol, and the mixture then evaporated to

dryness under a slow stream of nitrogen. The residue was dissolved in 2 ml methanol (100%), and an aliquot of 20 μl was used for analysis according to the method described by Sydenham et al. (1996) and Moses et al. (2010). For the HPLC analysis a 50-μl aliquot was used. To ensure reproducibility of fumonisin production *in vitro* and compare this to *in planta* fumonisin production, the *in vitro* experiment was repeated two more times with the same 15 *F. verticillioides* isolates that were selected for *in planta* studies.

The FB_1, FB_2 and FB_3 standards included in the HPLC analyses were obtained from the Mycotoxicology Research Group Institute of Biomedical and Microbial Biotechnology, Cape Peninsula University of Technology, Bellville, South Africa, and were all guaranteed 95% pure. These standards were all made up with acetonitrile/water (1:1), and the stock solution had a concentration of 1 g/l. Five working standards were made up from the stock solution with acetonitrile/water containing 2, 5, 10, 15 and 20 mg/kg (1:1). Fumonisin analogues were detected and quantified based on comparisons of retention times and peak area with standards (Rheeder et al. 2002). The detection limit of the method used was 0.016 mg/kg. Recovery data were obtained in triplicate by fortifying clean maize samples (VICAM) with 5 mg/kg fumonisin B_1, B_2 and B_3. The average recovery rates were 83% (FB_1), 81% (FB_2) and 83% (FB_3), respectively.

Virulence of *Fusarium verticillioides*

The maize cultivar CRN 3505 (susceptible to *F. verticillioides*) was used to determine the virulence of the same 15 *F. verticillioides* isolates that were selected for *in planta* fumonisin production. The plants were grown under dryland conditions (without irrigation) and managed according to standard farming practices. Stem borer control was performed by using Bulldock® 0.05 GR, which is a granular insecticide that is applied directly into the funnel of maize plants. Weed control was also performed using the pre-emergence herbicides Callisto® and Dual Gold®, and the post-emergence herbicide Basagran®. The trial was planted in a randomised block design, with three block replicates. Row lengths were 10 m and spacing of rows 90 cm. Maize ears were inoculated with a *F. verticillioides* spore suspension 1 week after silking, as described above. At harvest, the primary (inoculated) maize ears were handpicked, de-husked, and evaluated for severity of FER rot symptoms. Disease severity was assessed by determining the percentage of each ear covered by visible symptoms of infection, such as brown, pink or reddish discolouration of kernels, and pinkish or white mycelial growth (Clements et al. 2004; Small et al. 2012).

Statistical analysis

Statistical analysis to compare the virulence of *F. verticillioides* isolates was performed using SAS v9.2 statistical software (SAS 1999). Student's t-Least Significant Difference was calculated at the 5% level to compare treatment means for significant effects. When no significant differences were observed at the 5% level, data was log-transformed and Student's t-Least Significant Difference was calculated at the 10% level to compare treatment means and significant effects.

The data of the 15 *F. verticillioides* isolates tested in both experiments (*in vitro* and *in planta*) were tested for homogeneity of variances using Levene's test. The variability in the observations of the two experiments for total fumonisin was comparable, and analysis of variance could be validly carried out. Student's t-Least Significant Difference was calculated at the 5% level to compare treatment means of significant effects (SAS 1999). Pearson correlation coefficients were also calculated. Generally, a coefficient of ±0.7 and more is regarded as a fairly good correlation, whereas a correlation of ±0.9 indicates a very strong correlation. A correlation of ±0.5 is moderate, and a correlation of −0.3 to +0.3 is weak (Rayner 1969).

Results

Identification of isolates

Morphological identification A total of 415 *Fusarium* isolates were obtained from the roots (13), stems (42) and kernels (360) of maize collected in South Africa. Three *Fusarium* species were identified morphologically. These included *F. verticillioides*, *F. proliferatum* and *F. subglutinans*. The three species were separated based on macro- and microconidial characteristics. The microconidia of *F. verticillioides* were oval to club-shaped with a flattened base. Aerial mycelia were present as long chains, and the conidiogenous cells were monophialidic. Macroconidia were rare in culture for

F. subglutinans, and its microconidia were oval in shape. Conidia were produced in false heads on mono- and polyphialides. *Fusarium proliferatum* produced its club-shaped microconidia in chains of varying length on both mono-and polyphialides. There were 38 *Fusarium* isolates of which the identity could not be confirmed by morphological identification (Table 1).

Molecular identification Of the 415 *Fusarium* isolates obtained from maize, 291 isolates were confirmed as *F. verticillioides* (Table 2). Thirty-three isolates were confirmed as *F. subglutinans* by TEF1 gene sequencing, whereas 70 isolates were identified as *F. temperatum* Scauflaire and Munaut (Scauflaire et al. 2011a). Two isolates from maize kernels and one from a maize stem were identified as *F. andiyazi*, whereas isolates from maize roots and stems included two *F. nygamai* (roots), three *F. oxysporum* (kernel, stem and root, respectively) and nine *F. thapsinum* (stems) isolates. Only one isolate collected from kernels in Mpumulanga was identified as *F. proliferatum*, and one collected from kernels in the Eastern Cape Province as *F. bacterioides*.

Phylogenetic analysis Phylogenetic analysis of the *TEF*1 gene region separated *F. verticillioides* from the other *Fusarium* spp. found in maize plants with bootstrap values of 1000 for ML and of 1 for BI (Fig. 1). Each *Fusarium* sp. was divided into a separate lineage with above 0.9 ML and BI bootstrap values. This data confirmed the species specificity of the isolates collected from South Africa. *Fusarium verticillioides* isolates collected from maize kernels, stems and roots were divided into separate lineages, but without ML and BI support. There was no separation of *F. verticillioides* isolates into lineages according to geographical origin or levels of fumonisin being produced.

The eight *Fusarium* spp. isolated from maize kernels, stem and roots other than *F. verticillioides* were each divided into well-defined lineages. The

Table 1 The average total fumonisin production (mg/kg) measured using two methods namely, *in vitro* and *in planta*, by 15 *Fusarium verticillioides* isolates collected from maize kernels from resource-poor farmers and from maize stems and roots from commercial farmers in South Africa

Isolates	Locality	Province[a]	Plant Part	Total Fumonisin (mg/kg)[b]			
				in vitro		*in planta*	
GCI434	Giyani	LP	Kernel	21.32	a	14.04	abc
GCI441	Makhanisi	KZN	Kernel	16.23	ab	2.23	de
GCI2006	Wolmaranstad	NW	Root	5.06	dce	16.19	ab
GCI340	Jozini	KZN	Kernel	9.22	bcde	16.06	ab
GCI51	Idutywa	EC	Kernel	11.61	abcd	3.77	de
GCI1608	Ventersdorp	NW	Kernel	5.28	cde	11.18	bcd
GCI438	Venda	LP	Kernel	11.17	bcd	3.45	de
GCI445	Ermelo	MP	Kernel	2.34	de	9.36	bcde
MRC826	Transkei	EC	Kernel	7.87	bcde	4.45	cde
GCI309	Port Shepstone	KZN	Kernel	7.70	bcde	0.27	e
GCI297	Venda	LP	Kernel	4.07	cde	7.01	bcde
GCI77	Elundini	EC	Kernel	1.71	de	6.46	bcde
GCI2004	Wolmaranstad	NW	Stem	5.66	cde	4.85	cde
GCI2055	Wesselsbron	FS	Stem	5.17	dcde	2.48	de
GCI282	Pietersburg	LP	Kernel	0.00	e	1.86	de
Water	Control	NA		0.02	e	0.52	e

[a] Eastern Cape (EC), Mpumalanga (MP), KwaZulu-Natal (KZN), Limpopo (LP), North West (NW), Free State (FS) provinces

[b] Student's t-Least Significant Difference were calculated at the 5% level to compare treatment means of significant effects. A different letter in a column denotes significance

Table 2 The occurrence of *Fusarium* spp. in the different provinces in South Africa as identified with sequencing of the translation elongation factor-1a (TEF1) gene region sequence

	Province	Eastern Cape	KwaZulu-Natal	Mpumalanga	Limpopo	North West			Free State			Total
	Plant part	Kernel	Kernel	Kernel	Kernel	Kernel	Stem	Root	Kernel	Stem	Root	
Fusarium spp.	*F. verticillioides*	58	97	48	35	9	16	2	5	14	7	291
	F. temperatum	40	8	22								70
	F. subglutinans	20	7	3	3							33
	F. thapsinum						9			1		9
	F. oxysporum				1		1	1			1	4
	F. andiyazi			1	1		1					3
	F. proliferatum			1								1
	F. nygamai							2				2
	F. bacterioides	1										1
	Total	119	112	75	40		39			28		413

most noticeable of these were *F. subglutinans* and *F. temperatum* isolates obtained from maize kernels that grouped together as two closely related lineages with high ML and BI support. *Fusarium andiyazi* isolates formed a separate lineage with bootstrap values of ML of 1000 and BI of 1, while three *F. oxysporum* isolates from maize roots and stems grouped together to serve as root for the phylogenetic tree.

Fumonisin production by *Fusarium verticillioides* isolates

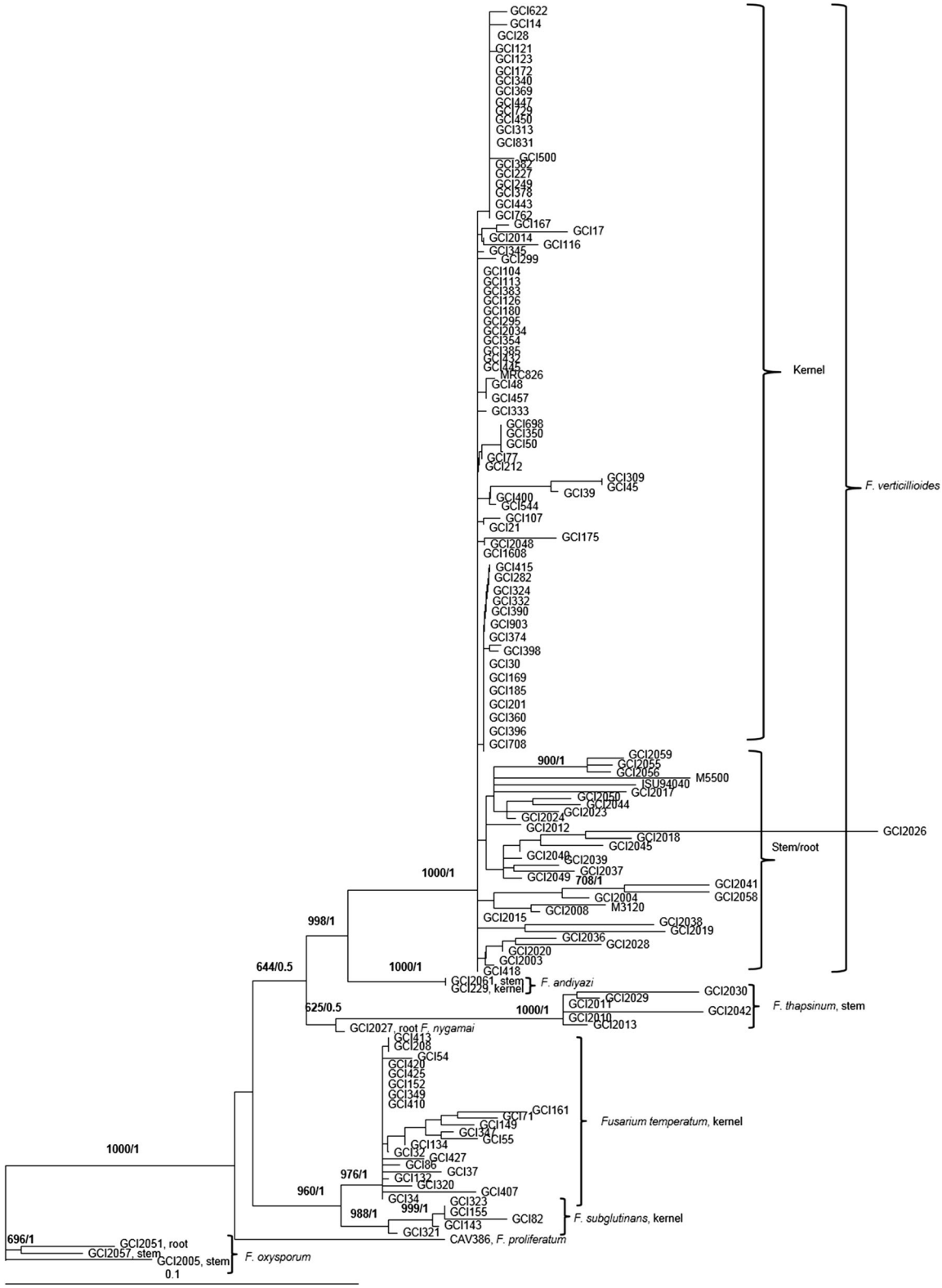
Of the 291 *F. verticillioides* isolates tested *in vitro*, 160 isolates produced fumonisin levels between 0 and 2 mg/kg (Table 3). Seventeen isolates produced no fumonisins, while nine isolates produced fumonisin levels in excess of 20 mg/kg. These isolates were obtained from maize kernels collected in rural areas in the Limpopo (1), Mpumalanga (2), Eastern Cape (3) and KwaZulu-Natal (3) provinces. All *F. verticillioides* isolates collected in Mpumalanga produced detectable levels of fumonisins.

In vitro vs *in planta* analysis of fumonisin

The *F. verticillioides* isolates differed significantly regarding main effects on fumonisin production levels ($P = 0.00$). However, there was a significant two-way interaction between methods (*in vitro* or *in planta* evaluation of fumonisin produced) and *F. verticillioides* isolates tested ($P = 0.04$) (Table 4).

Isolate GCI 434 (21.32 mg/kg), which was collected from maize kernels in Giyani, produced more fumonisin *in vitro* than any of the other *F. verticillioides* isolates (Table 1). Its production, however, was not significantly more than that of isolate GCI 441 (16.23 mg/kg), which was collected from maize kernels in Makhanisi in KwaZulu-Natal, and isolate GCI 51 (11.61 mg/kg), which was collected from maize kernels in Idutywa, Eastern Cape Province (Table 1). GCI 434 also produced most fumonisins in the *in planta* trial, along with isolates GCI 2006 (16.19 mg/kg) and GCI 340 (16.06 mg/kg). GCI 282 was the only isolate to produce less than 2 mg/kg both *in vitro* and *in planta*.

High fumonisin production by GCI 434, as well as the low fumonisin production by GCI 1282, did not differ significantly when measured *in vitro* and *in planta* (Table 1). Fumonisin production by GCI 282 also did


GCI622
GCI14
GCI28
GCI121
GCI123
GCI172
GCI340
GCI369
GCI447
GCI450
GCI313
GCI831
GCI500
GCI227
GCI378
GCI443
GCI762
GCI167
GCI17
GCI2014
GCI116
GCI345
GCI299
GCI104
GCI113
GCI383
GCI126
GCI180
GCI295
GCI2034
GCI354
GCI385
MRC826
GCI48
GCI457
GCI333
GCI698
GCI350
GCI50
GCI77
GCI212
GCI309
GCI45
GCI39
GCI400
GCI544
GCI107
GCI21
GCI175
GCI2048
GCI1608
GCI415
GCI282
GCI324
GCI332
GCI390
GCI903
GCI374
GCI398
GCI30
GCI169
GCI185
GCI201
GCI360
GCI396
GCI708
Kernel
F. verticillioides
900/1
GCI2059
GCI2055
GCI2056
M5500
ISU94040
GCI2017
GCI2050
GCI2044
GCI2023
GCI2024
GCI2012
GCI2026
GCI2018
GCI2045
GCI2040
GCI2039
GCI2037
GCI2049
Stem/root
1000/1
708/1
GCI2041
GCI2058
GCI2004
M3120
GCI2008
998/1
GCI2015
GCI2038
GCI2019
GCI2036
GCI2028
GCI2020
GCI2003
GCI418
644/0.5
1000/1
GCI2061, stem
GCI229, kernel
F. andiyazi
GCI2030
GCI2029
GCI2011
GCI2042
F. thapsinum, stem
625/0.5
1000/1
GCI2010
GCI2013
GCI2027, root F. nygamai
GCI413
GCI208
GCI54
GCI420
GCI425
GCI152
GCI349
GCI410
GCI161
GCI71
GCI149
GCI341
GCI55
GCI134
GCI32
GCI427
GCI86
GCI37
976/1
GCI132
GCI320
960/1
GCI407
GCI34
GCI323
999/1
GCI155
GCI82
988/1
GCI143
GCI321
Fusarium temperatum, kernel
F. subglutinans, kernel
1000/1
CAV386, F. proliferatum
696/1
GCI2051, root
GCI2057, stem
GCI2005, stem
F. oxysporum
0.1

◀ **Fig. 1** A phylogenetic tree constructed for 139 *Fusarium vertcillioides* isolates and 44 *Fusarium* spp. from maize kernels, roots and stems in South Africa using the translation elongation factor-1a (TEF1) gene region sequences. The tree was drawn using the TPM3 + G evolution model

not differ significantly from the water control. When *in vitro* and *in planta* fumonisin production of other *F. verticillioides* isolates were compared, GCI 441 produced significantly more fumonisins *in vitro* (16.23 mg/kg) than *in planta* (2.23 mg/kg) and GCI 2006 produced significantly higher fumonisin levels *in planta* (16.17 mg/kg) than *in vitro* (5.06 mg/kg) (Table 1). Although differences in fumonisin levels were measured *in vitro* and *in planta* for the other *F. verticillioides* isolates tested, these differences were not significant ($P \leq 0{,}05$). There was, however, a weak correlation between *in vitro* produced fumonisin levels and in vivo fumonisin levels produced by the different *F. verticillioides* isolates ($P = 0.160$; $R^2 = 0.043$).

Virulence of *Fusarium verticillioides*

All *F. verticillioides* isolates tested for pathogenicity to maize ears were able to produce FER symptoms. Disease severity, however, was low, with mean ear rot symptoms not greater than 3%. No significant differences were found among the isolates at $P < 0.05$ and $P < 0.1$ (results not presented).

Discussion

Endophytic and pathogenic *F. verticillioides* isolates collected from maize ears, stems and roots in this study belonged to a single *Fusarium* species. *Fusarium verticillioides* is well-known to be associated with the crop both as endophyte and pathogen (Yates et al. 1997). Despite differences in origin, virulence to maize and fumonisin production, isolates of *F. verticillioides* in South Africa represent a single morphological and phylogenetic species that grouped separate from other *Fusarium* species associated with maize with strong ML or BI support.

The ability of *F. verticillioides* isolates to produce fumonisins appears to be unrelated to the geographical region where they were collected from in South Africa. *Fusarium verticillioides* isolates in Brazil also did not

Table 3 The number of *Fusarium verticillioides* isolates collected from maize kernels, stem and roots from subsistence and commercial farmers in seven provinces in South Africa

Fumonisin concentration	Production by commercial growers						Production by subsistence growers						Total (South Africa)
	Free State			North West			Eastern Cape		Limpopo		Mpumalanga	KwaZulu-Natal	
	Kernel	Stem	Root	Kernel	Stem	Root	Kernel		Kernel		Kernel	Kernel	
0 mg/kg	1	2	1	2	1		4		3			3	17
0–2 mg/kg	4	10	5	4	6	1	29		17		30	54	160
2–5 mg/kg		1	1	1	7		10		8		7	11	45
5–10 mg/kg		1		2	2	1	4		3		4	19	35
10–20 mg/kg							8		3		6	7	25
>20 mg/kg							3		1		2	3	9
Total	26			27			58		35		48	97	291

Table 4 ANOVA with probabilities of F-ratios for total fumonisin production using two methods namely, *in vitro* and *in planta*, by 15 *Fusarium verticillioides* isolates

Total fumonisin (mg/kg)			
Sources of variation	Degrees of freedom.	Mean square	F.pr.
Method[a]	1	8.64	0.63
Isolate[b]	15	115.55	0.00[c]
Method x Isolate	15	70.92	0.04[c]

[a] Toxin production method *in vitro* vs. *in planta*

[b] *Fusarium verticillioides* isolates

[c] Significant difference at $P \leq 0.05$ according to student's t-LSD

group according to fumonisin production or geographical area (De Oliveira Rocha et al. 2011). This was also the results found by using *F. verticillioides* isolates from maize kernels from Argentina, China, Italy, Portugal, Spain and South Africa (Mirete et al. 2004). In contrast, Cumagun et al. (2009) found that *F. verticillioides* isolates from northern Luzon in the Phillipines produced higher fumonisin levels than isolates from the southern part of Luzon. Fumonisin analysis was performed *in vitro*, thereby indicating the importance of the genetics in fumonisin production in the Philippines.

Fumonisin production by *F. verticillioides* is influenced by temperature, incubation temperature, duration of culturing *in vitro* (Alberts et al. 1990; Melcion et al. 1997; Jiménez et al. 2003; Vismer et al. 2004), drought stress, the combination of sugar and amino acids of maize varieties and time of harvest *in planta* (Miller 2001; Jiménez et al. 2003; Bush et al. 2004; Parsons and Munkvold 2012). In this study, however, *in vitro* experiments were performed under stable laboratory conditions, whereas field trials were performed at a single locality where environmental conditions were similar for all plants included in the experiment. Thirteen of the 15 isolates tested produced fumonisins levels that did not differ significantly *in planta* and *in vitro*. The variation in fumonisin levels found thus reflected the toxigenic potential of individual isolates. Some isolates, such as GCI 441 and GCI 2006, produced fumonisin at levels that differed when tested *in vitro* and *in planta*. It is possible that these *F. verticillioides* isolates varied in their regulation of fumonisin production *in vitro* and *in planta* due to differences in the source of carbon in the two substrates (Jiménez et al. 2003; Vismer et al. 2004). The regulation of fumonisin production is controlled by FUM genes. López-Errasquín et al. (2007), for instance, has shown that differences in fumonisin production by *F. verticillioides* under laboratory conditions were reflected by differences in the expression of FUM1.

A *Fusarium* species newly identified on maize ears in South Africa was *F. temperatum*. This fungus was previously designated *F. subglutinans*, but Scauflaire et al. (2011a) renamed isolates able to produce beauvericin as *F. temperatum*, whereas isolates of *F. subglutinans* that produce only moniliformin retained the original name (Moretti et al. 2008). *Fusarium temperatum* is the more dominant species associated with maize in South Africa when compared to *F. subglutinans*. Similar to *F. subglutinans*, *F. temperatum* was found in the cooler areas of Mpumalanga, the Eastern Cape and the mountainous regions of KwaZulu-Natal. Maize consumed as food and feed in these provinces can thus be contaminated with beauvericin. This will be important as beauvericin toxin induces programmed cell death in mammals. The ability of these *F. temperatum* isolates to produce beauvericin should be considered in future studies in order to prevent misclassification.

Fusarium andiyazi, *F. nygamai* and *F. thapsinum* were infrequently associated with maize kernels, roots and stems in South Africa. They are, however, morphologically similar to *F. verticillioides* and can therefore be confused during morphological identification. *Fusarium nygamai* and *F. andiyazi* produce fumonisin and moniliformin, whereas *F. thapsinum* produces moniliformin only (Leslie et al. 2005). *Fusarium oxysporum*, a soil-borne pathogen commonly associated with maize root and stalk rot (White 1999), was isolated from root and stem tissue in the North West and Free State provinces. The occurrence of these *Fusarium* species in South African maize is most likely due to farming practices such as rotation with other crops such as sorghum or planting of sorghum with maize on the same fields, while *F. oxysporum* is universally present in soil. The single *F. proliferatum* isolate found on maize grain in this study indicated its insignificance as maize pathogen in the country (Rheeder et al. 1992; Boutigny et al. 2012), despite its importance in Europe as ear rot pathogen along with *F. verticillioides*, *F. subglutinans* and *F. graminearum* (Goertz et al. 2010; Scauflaire et al. 2011b).

This study investigated the variation in virulence and fumonisin production of *F. verticillioides* isolates from maize kernels, stems and roots in South Africa. The FER fungus could neither be divided into groups according to fumonisin produced, nor show any

preference for geographic origin or plants tissue. The fumonisin-production ability of each *F. verticillioides* isolate was responsible for similar fumonisin levels producec *in vitro* and *in planta*. All *F. verticillioides* isolates tested were able to cause FER, thereby proving their ability to cause disease to maize ears despite the tissue they were collected from. This study was the first to confirm the presence of high numbers of the beauvericin-producing fungus *F. temperatum* in maize ears in South Africa.

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