First report of *Fusarium boothii* from pecan (*Carya illinoinensis*) and camel thorn (*Vachellia erioloba*) trees in South Africa

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Abbreviations:

15-ADON, 15- acetyl-Deoxynivalenol; FCSC, *Fusarium chlamydosporum* species complex; FGSC, *Fusarium graminearum* species complex; FHB, Fusarium head blight; FOSC, *Fusarium oxysporum* species complex; GER, Gibberella ear rot; PDA, Potato Dextrose Agar; TEF, Translation Elongation factor 1-α.
ABSTRACT

*Fusarium boothii* forms part of the *Fusarium graminearum* species complex (FGSC), the important grain pathogen group that causes Gibberella ear rot of maize and *Fusarium* head blight of wheat. It is known to infect many grain crops such as maize, wheat and barley. Moreover, this pathogen is a 15-ADON mycotoxin producer and thus of concern for stored grains and grain products. During endophyte isolations in the Hoopstad area of the two unrelated trees, pecan and camel thorn, isolates of the FGSC constituted of a dominant part of the *Fusarium* isolates obtained. Pecan (*Carya illinoinensis*) is a rapidly developing industry in the semi-arid to arid regions of South Africa, while camel thorn (*Vachellia erioloba*) is a dominant native tree in the same areas. DNA sequence comparisons of the Translation Elongation Factor 1-α and β-tubulin gene regions clearly showed that these isolates were *F. boothii*. This study thus represents the first report of this grain pathogen from unlikely tree hosts, and only the second report of a species in the FGSC from trees, the other being that of *Fusarium acacia-mearnsii*. The unexpected occurrence of *F. boothii* in other type of hosts than grain could represent an important gap in our understanding of the epidemiology, geographical occurrence and movement, and genetic pool of this important pathogen. It also raises the question whether other species in the FGSC could have unexpected host associations.

1. **Introduction**

Fusarium head blight (FHB) is a disease of wheat (*Triticum aestivum* L.) where florets or entire spikes of the wheat plant are infected, giving them a bleached
appearance or discolouration at the base of the head (Goswami and Kistler, 2004).

Individual kernels may become shriveled and white (Goswami and Kistler, 2004).

This disease already had several serious outbreaks globally, and causes yield loss and
low test weights, low seed germination and contamination of grain with mycotoxins
(Goswami and Kistler, 2004). It is a major research focus for the international wheat
industry, and also affects barley (Hordeum vulgare L.), oats (Avena sativa L.), maize
(Zea mays L.), and rice (Oryza sativa L.) (Goswami and Kistler, 2004). It is caused
by several spp. of Fusarium such as F. avenaceum (Fr.) Sacc., F. poae (Peck)
Wollenw. and F. sporotrichioides Sherb., with F. graminearum Schwabe s. l., F.
culmorum (W.G. Sm.) Sacc., and F. cerealis (Cooke) Sacc. (= F. crookwellense L.W.
Burgess, P.E. Nelson & Toussoun) the most prominent pathogens (Kotowicz et al.,
2014). However, the past couple of years showed a major increase in dominance of
F. graminearum s.l. (Goswami and Kistler, 2004).

On maize, F. graminearum s.l. causes a disease known as Gibberella ear rot
(GER) (Boutigny et al., 2012; Munkfold, 2003; Sampietro et al., 2010, 2012). This is
due to the occurrence of the sexual state of F. graminearum, namely G. zeae, on the
symptoms (Boutigny et al., 2012; Munkfold, 2003). The fungus colonizes the cob
from the tip and rot progresses downward (Munkfold, 2003). Similarly to FHB,
various mycotoxins are produced in the cob that are dangerous to those consuming the
cobs or derived food.

The F. graminearum species complex (FGSC) consists of 15 described species
contributing to FHB and GER in various parts of the world (Aoki et al., 2012). Of
these, F. graminearum s. str. is the species most commonly associated with disease
(Goswami and Kistler, 2004). Although morphologically indistinguishable, the
species can be separated by twelve phylogenetic markers (Aoki et al., 2012). Some
species such as *F. graminearum s. str.* occurs globally, while others have only been reported from single countries such as *F. aethiopicum* O'Donnell, Aberra, Kistler & T. Aoki (Aoki et al., 2012). The various species also have certain unique chemotypes whereby they have the genetic potential to produce different combinations of mycotoxins (Aoki et al., 2012).

A number of studies has started to elucidate the distribution of members of the FGSC throughout South Africa on grain crops such as wheat, barley and maize (Boutigny et al., 2011a, 2011b, 2012; Lamprecht et al., 2011). These have shown that only six species (*F. graminearum s. str.*, *F. meridionale* T. Aoki, Kistler, Geiser & O'Donnell, *F. acaciae-mearnsii* O'Donnell, T. Aoki, Kistler & Geiser, *F. brasilicum* T. Aoki, Kistler, Geiser & O'Donnell, *F. boothii* O'Donnell, T. Aoki, Kistler & Geiser and *F. cortaderiae* O'Donnell, T. Aoki, Kistler & Geiser) occur in South Africa. Rudimentary geographical patterns of these species may exist, and a level of host preference to certain crops has been observed. Of these, *F. acaciae-mearnsii* is the only species that occurs on a different type of host other than cereals, namely wattle (*Acacia mearnsii* De Wild.) and eucalypt (*Eucalyptus grandis* W. Hill ex Maiden) trees (Aoki et al., 2012).

In the past surrounding native vegetation, or naturalized or invasive non-native plants, have been shown to play important roles in the epidemiology of diseases, where these are often alternative hosts where the pathogens can survive, increase in numbers in the absence of the known host plants or generate additional genetic diversity. Absence of such data impacts on our understanding of the current geographical distribution and possible movements of pathogens, and their potential to become genetically more diverse on these hosts. This could negatively affect disease management programmes and mycotoxin risk assessments.
North-western areas of South Africa range from arid to semi-arid. These areas are planted with agricultural crops such as maize, wheat, sunflowers, potatoes and groundnut. Few tree crops are grown in these areas due to lack of water, with pecan nuts \textit{[Carya illinoiensis} (Wangenh.) K. Koch] one of the few. Pecan is rapidly becoming a large industry in South Africa (Erasmus, 2011). Not many serious diseases are as yet published from this crop. Vegetation surrounding crops in the North-western areas of South Africa comprise of grassland and predominantly \textit{Vachellia} and \textit{Senegalia} tree species, which was known as \textit{Acacia} in the past (Kyalangalilwa et al., 2013). Of these, \textit{Vachellia erioloba} (E. Mey.) P. J. H. Hurter (camel thorn), previously named \textit{Acacia erioloba} E. Mey. (Kyalangalilwa et al., 2013), is the dominant form.

A pilot survey was done in the Hoopstad area of South Africa (Free State province) to investigate the co-infection of fungal endophytes and latent pathogens between non-native pecan trees and native \textit{V. erioloba}. Endophytes are organisms living asymptomatically inside tree tissues, while latent pathogens are plant pathogens that have an endophytic phase (Slippers and Wingfield, 2007). The presence of pathogens known to infect the crops in the area was also investigated in these unrelated trees. A number of \textit{Fusarium} isolates were obtained from these trees during the survey. \textit{Fusarium} are well known fungi that include devastating plant pathogens of various crops, mycotoxin producers and also pathogens of humans (Leslie and Summerell, 2006). What was of special interest was a number of isolates morphologically resembling species in the \textit{Fusarium graminearum} species complex (FGSC). The aim of this study was to determine which species in the complex these isolates represent and if they include pathogens known from South Africa. Possible disease reactions on pecan leaves were also assessed in bioassays.
2. Material and methods

2.1 Collection of samples

Ten trees of *V. erioloba* and pecan, respectively, were sampled at two sites about 500 m apart in the Hoopstad area, Free State. Ten branches were randomly selected from each tree, and ten leaves were cut from each branch. Ten pieces per leaf and per branch (c. 4 mm diam.) were plated onto 2% Potato Dextrose Agar (Biolab, Merck Millipore, South Africa) after surface sterilization (1 min wash with sterile water, 5 min submersion in 3% sodium hypochloride followed by 5 min in 70% ethanol, final rinse in sterile water). Resultant colonies were purified onto PDA plates and identified morphologically. Isolates resembling *Fusarium* spp. were single spored and maintained on PDA. Representative isolates were deposited in the National Collection of Fungi, Agricultural Research Council, Pretoria, South Africa.

2.2 Identification with DNA sequence comparisons

DNA was extracted from scraped mycelium of six representative and morphologically distinct seven-day-old cultures (Fig. 1) using the method developed by Möller et al. (1992). PCR amplicons and sequences of the Translation Elongation Factor 1-α and β-tubulin genes were obtained following the protocols of O’Donnell et al. (2000, 2004) using the Robust PCR kit (KAPA Biosystems). Amplification products were visualized on 1% agarose gels (Cleaver Scientific, AEC-Amersham, South Africa) containing Gelred DNA stain (Biotium, Anatech, South Africa) under UV illumination using a Geldoc XR+ imaging system (Bio-Rad, South Africa).
Up to 20 ng/µl of PCR amplicons, purified using the EXO/SAP Amplicon Purification system (Werle et al., 1994), were used for sequencing reactions with the BigDye Terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were purified with EDTA/Ethanol precipitation and run on an ABI 3130XL genetic analyzer (Applied Biosystems). Chromatograms were compiled into contigs with Geneious v. 7.0.6 (Biomatters, New Zealand) and the DNA sequences were submitted to Genbank (TEF 1-α: KU325471, KU325473- KU325474, KU325476, KU325479, KU325482; β-tubulin genes: KU325458, KU325461- KU325462, KU325465, KU325467, KU325469). Generated sequences were added to datasets of the TEF1-α and β-tubulin genes for the FGSC in Mega 6.06 (Tamura et al., 2013) obtained from Dr Kerry O’Donnell (United States Department of Agriculture). Maximum likelihood analyses with 1000 bootstrap replicates were done on the datasets with MEGA 6.06 using appropriate models also obtained with MEGA. Initial analyses were done separately on the TEF1-α and β-tubulin datasets, but the datasets were combined since both gave congruent phylogenetic trees and were proven previously to be combinable (O’Donnell et al., 2000, 2004).

2.3 Bioassays

Two repeats of fresh leaf bioassays (Keith and Zee, 2010) with five isolates (Fig. 2) in the FGSC were done on fresh, mature pecan leaves to test if the isolates had an ability to cause lesions on this host. Similar assays on V. eriobotrya leaves proved too difficult due to the small size of the leaf pinnules of the compound leaves. Agar blocks (4 mm diam) with mycelium were placed on 10 surface sterilized leaves for each isolate at wounds created by a sterile needle. Clean agar blocks were used for negative
controls. Leaves were kept in moist chambers at room temperature and leaf lesions were scored after three weeks (1=no lesion, 2=small lesion, 3=intermediate lesion, 4=lesion covered leaf). The average scale for each isolate and the negative control were calculated and represented as a graph.

3. Results

3.1 Collection of samples

The number of *Fusarium* isolates (33) was small relative to the c. 2000 cultures of fungi isolated from the trees. Of the 18 representatives sequenced, 12 represented the FGSC, while the others were shown to be in the *F. chlamydosporum* species complex (FCSC) and *F. oxysporum* species complex (FOSC) (data not shown). The FGSC isolates originated from both pecan and *V. erioloba* (one isolate from *V. erioloba* and 11 from pecan for the FGSC), while two isolates from *V. erioloba* and one from pecan grouped in the FCSC, and three from *V. erioloba* in the FOSC.

3.2 Identification with DNA sequence comparisons

The combined dataset including TEF1-α and β-tubulin DNA sequences consisted of 71 taxa and 991 characters. In the FGSC, the isolates grouped with *F. boothii* (Bootstrap support 84%) based on TEF1-α and β-tubulin DNA sequences (Fig. 1). This is different from results of the Brazilian isolates that grouped with *F. graminearum s. str.* and *F. austroamericanum*, similar to results from Lazarotto et al. (2014a).

3.3 Bioassays
All isolates of the FGSC tested formed lesions in the bioassays (Fig. 2-3). No lesions formed in the control inoculations. The degree of lesions formed varied between isolates with some completely consuming leaves (Fig. 3). The inoculated fungi could be re-isolated from the lesions and their identity were confirmed using DNA sequencing.

### 4. Discussion

This study reports the presence of the cereal pathogen *F. boothii* from tree hosts for the first time. These trees include the common native tree *V. erioloba*, and the non-native and unrelated tree crop pecan. This study also indicated that *F. boothii* co-infected these two unrelated hosts where they co-occur. *F. boothii* is only known from barley, maize and wheat in several countries in Europe, South America, the U.S.A. and South Africa (Boutigny et al., 2011a, 2011b, 2014; Desjardins and Proctor, 2009; Malihipour et al., 2012; Sampietro et al., 2010, 2012; Tóth et al., 2005) and has never before been reported from a native plant. Two other different types of hosts include soybean [*Glycine max* (L.) Merr.] (Chiotta et al., 2015) and tomato (*Solanum lycopersicum* L.) (Gomes et al., 2015).

The fact that the important pathogen *F. boothii* was found in trees has important implications for the epidemiology of the disease *F. boothii* causes on barley, maize and wheat. Although the maize fields adjacent to the pecan and *V. erioloba* trees were not sampled during the survey, it is likely that *F. boothii* occurred in these fields as it is known from maize, wheat and barley throughout the Free State, North West and Northern Cape (Boutigny et al., 2011a, 2011b, 2012). It is not yet known what other hosts it could infect, and on what it may be present in the Hoopstad area, including other agricultural crops such as sunflowers and groundnut. The host
range of *F. boothii* most likely is much wider than anticipated if the fungus can infect such unrelated and physiologically different hosts. These alternative hosts represent unrecognized sources of pathogen inoculum for the next season of growth, could potentially be sources where the fungus could generate genetic variation developing more virulent and toxigenic strains, or be asymptomatic vessels with which to move the pathogen (Swett and Gordon, 2015). Widely occurring *V. erioloba* trees could also represent a natural corridor connecting different populations of the pathogen between cultural lands and areas.

*Fusarium acacia-mearnsii* was described from the non-native trees *A. mearnsii* and *E. grandis* in the KwaZulu-Natal province, South Africa (O’Donnell et al., 2004). This fungus was able to cause lesions on these trees during artificial inoculations, and was associated with lesions in the field (Roux et al., 2001). During national surveys on wheat, maize and barley, this species was detected from wheat in KwaZulu-Natal, and it is able to infect a cereal naturally (Boutigny et al., 2011a). It is thus also able to infect such different types of hosts as has been found for *F. boothii* in this study. It would have been expected that *F. acacia-mearnsii* was a likely candidate to infect *V. erioloba* and pecan in this study, but instead another member of the FGSC was detected. It could be possible that the geographic range of *F. acacia-mearnsii* does not yet include the Hoopstad area in South Africa, as previous studies only linked it to Kwazulu/Natal (Roux et al., 2001; Lamprecht et al., 2011; Boutigny et al., 2011).

There are few significant diseases caused by *Fusarium* spp. from pecan. *F. equiseti* has been isolated from seedling necrosis, wilt and root rot symptoms in Brazil and were shown to be pathogenic (Lazarotto et al., 2014b), while *F. solani* was shown to cause root necrosis (Hsu and Hendrix, 1973). *Fusarium* species of the *F.*
chlamydosporum species complex, *F. oxysporum* species complex, *F. proliferatum*, and *F. austroamericanum* and *F. graminearum s. str.* of the *F. graminearum* species complex were isolated from diseased pecan in Brazil (Lazaretto et al., 2014a).

Bioassays from this study indicated that the endophytically isolated *F. boothii* can cause lesions on pecan leaves, but more extensive surveys will be necessary to determine if *F. boothii* is really associated with any naturally occurring disease symptoms on this host.

Another concern that must be investigated, is the association of *F. boothii* with pecan nuts. *F. boothii* produces 15-ADON trichothecenes that poses a serious health hazard (Aoki et al., 2012). Our study did not include isolations from pecan nuts, but it must be determined if this fungus can infect and survive in nuts either in the field or during storage, and if it will produce significant levels of mycotoxins. Very few and especially recent studies report on the infection and mycotoxins levels of pecan nuts (Huan and Hanlin, 1975; Terabe et al., 2008).

Only one member of the FGSC is primarily known from trees, i.e. *F. acacia-mearnsii* (Aoki et al., 2012; Roux et al., 2001), while the rest are predominantly known from cereals. Other types of hosts for members of the FGSC include vine, pampas grass, carnations, giant cane, fern, banana, grape ivy, and soil (Aoki et al., 2012). However, *F. graminearum s. str.* and *F. austroamericanum* have been reported from pecan in Brazil (Lazaretto et al., 2014a), together with *F. boothii* from this study. *F. meridionale* has been found from an orange twig (Aoki et al., 2012), but it is unknown if the fungus occurs naturally on this tree. The presence of four species in the FGSC on woody hosts besides cereals, raises the question if other species in the FGSC could also have such wide and diverse host ranges. Such unexpected host associations has been shown for the important pine pathogen *F. circinatum* that has
also been shown to naturally occur on maize and grasses, a fact that sheds new light on its evolutionary development and epidemiology (Swett and Gordon, 2009; 2012; 2015; Swett et al., 2014).

The aims of disease management programmes against FHB and GER include the prediction of disease threats and the potential mycotoxin contamination risk (Sampietro et al., 2010). Knowledge of the current species composition and substrate or host associations in a particular area facilitates this because the various species in the FGSC produces known mycotoxins and have fairly well-studied plant health risks. If there are unprecedented hosts and niches of these pathogens in an area that could harbor problem species, this would compromise these management efforts. This is especially so when there are no typical symptoms when the fungi occur asymptomatically, as was the case in this study. Inadvertent movement of species, genetic diversity of species already known to occur in the area, or new chemotypes of species that can produce other mycotoxins, could also be produced on these unexpected hosts, or they could be natural corridors of movement overlooked when only human-mediated movement are taken into account.

It must be determined if the occurrence of *F. boothii* on pecan and a native *Vachellia* species is significant. Unexpected hosts can be refugia for this pathogen that is thought to occur predominantly on grass-like crops. It is known that species in the FGSC can survive on plant debris in the winter (Goswami and Kistler, 2004) and plant matter from these new hosts could provide additional inoculum. The extent of such co-infection could be localized around particular, already infected fields, or perhaps be more extensive and naturally occurring. Hypotheses on evolution, origin, movement, and possible sources of infection are largely based on research from agricultural, food, veterinarian and human substrates. Therefore, the occurrence of
known species from unexpected hosts, substrates and niches influences our understanding of these problem fungi. Such questions would have to be pursued with more extensive and targeted sampling.

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Figure legends:

Fig. 1. Maximum likelihood tree of the *Fusarium graminearum* species complex based on the TEF-1α and β-Tubulin genes. Bootstrap values (1000 replicates) are indicated on the branches. *F. lunolusporum, F. cerealis, F. culmorum* and *F. pseudograminearum* are the outgroup taxa.

Fig. 2. Lesion scores for pecan leaves inoculated with *Fusarium boothii* isolates from pecan and camel thorn. (1=no lesion, 2=small lesion, 3=intermediate lesion, 4=lesion covered leaf)

Fig. 3. A variety of lesions on inoculated leaves from the bioassay. (a) Negative control (no lesions). (b) *F. boothii* isolate “By” (indicated by arrows).
Figure 1

- F. graminearum
- F. mesoamericanum
- F. asiaticum
- F. vorosii
- F. vorosii
- F. ussurianum
- F. aethiopicum
- F. acaciae-mearnsii
- F. gerlachii
- F. louisianense
- F. meridionale
- F. australiamericanum
- F. brasilicum
- F. cortaderiae
- F. boothii
Figure 2

Lesion score

| Isolates   | Lesion scores |
|------------|---------------|
| Control    | 0             |
| PPRI 19334 | 1             |
| PPRI 19346 | 2             |
| PPRI 19347 | 3             |
| PPRI 19336 | 4             |
| PPRI 19335 | 4             |
