INTERACTIVE EFFECT OF *BUSSEOLA FUSCA* AND *FUSARIUM VERTICILLIOIDES* ON EAR ROT AND FUMONISIN PRODUCTION IN MAIZE

by

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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of AgriSciences at the University of Stellenbosch

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March 2017

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

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SUMMARY

Maize is a crop of great economic importance in southern Africa, and is widely consumed as a staple food and animal feed. Production of maize, however, is hampered by pathogens and pests such as *Fusarium verticillioides* and the African stem borer *Busseola fusca*, respectively. *Fusarium verticillioides* infection results in Fusarium ear rot (FER) and contamination of maize kernels with fumonisin mycotoxins, while *B. fusca,* causes significant damage to maize tissues during larval feeding. Despite attempts to control *F. verticillioides*, fungal infection and fumonisin production remains a threat to maize production due to a lack of resistant maize cultivars and the inability to target the pathogen with fungicides and biocontrol products. Planting *Bt* maize hybrids have become an important mechanism for the management of stem borers of maize. However, the recent discovery of *B*. *fusca* resistance to *Bt* maize with a single crystal protein *MON810* gene, indicates that care should be taken not to solely rely on this technology for the management of *B. fusca*.

The interactive effect of *B. fusca* and *F. verticillioides* on FER and fumonisin production in maize was investigated in this study. Maize ears were inoculated with *F. verticillioides* alone, with both *F. verticillioides* and *B. fusca*, and with *B. fusca* alone. *Fusarium verticillioides* isolate MRC826 was inoculated by injecting a spore suspension of the fungus into the silk channel of each primary ear at the blister stage. For *B. fusca* infestation, aliquots of 10-15 neonate larvae were deposited into the whorl of each plant at the 12-13th leaf stage before tasselling using a mechanical applicator. Maize ears were also mechanically wounded at the blister stage with a cork borer (different sizes and number of wounds) to mimic hail damage, and half of the wounds infected with *F. verticillioides*. Results from this study indicated that the impact of *B. fusca* infestation on FER varied seasonally, possibly due to its sporadic damage to maize ears. *Busseola fusca*, however, did not result in a significant increase in fumonisin production. The severity of wounding of maize ears was an important contributor to FER development and fumonisin production.

The effect of host plant genetic modification and pesticide application on FER and fumonisin production in maize was investigated by studying the response of a *Bt* hybrid and its non-*Bt* isohybrid to *F. verticillioides* infection and *B. fusca* infestation; and by treating plants with Beta-cyfluthrin (non-systemic) and Benfuracarb (systemic) insecticides. The field trials were conducted over three seasons using a randomised complete block design with six replicates per treatment. Uninoculated, uninfested and undamaged control treatments were included. All ears were harvested at physiological maturity and FER, total fumonisin concentration, stem borer cumulative tunnel length (*B. fusca* damage) and target DNA of fumonisin-producing *Fusarium* spp. were quantified. *Busseola fusca* infestation had no effect

on fungal colonisation and fumonisin production in maize. *Bt* and non-*Bt* kernels were equally contaminated with fungal DNA, but FER and fumonisin production were reduced in the *Bt* hybrid under natural farming conditions. Despite the evidence found in this study and others that *Bt* maize indirectly reduces FER and fumonisin production, this was also inconsistent over seasons. Benfuracarb controlled stem borers, and therewith indirectly reduced FER and fumonisin production. FER development and fumonisin production by *F. verticillioides* varied over seasons, indicating the importance of environmental conditions on FER and fumonisin production.

A survey was also conducted at two sites in the North West province and one site in the Free State province of South Africa to analyse mycoflora in *B. fusca* frass. The exposure of *B. fusca* larvae to *F. verticillioides* in stem borer frass was also evaluated in both greenhouse and field trials. Maize whorls were inoculated with a spore suspension of *F. verticillioides* MRC826 4 weeks after plant emergence and infested with aliquots of 5-10 neonate *B. fusca* larvae 2 days later. The control treatment consisted of *B. fusca* infestation only. Several fungal species were associated with stem borer frass, including *Acremonium zeae*, *Aspergillus flavus*, *A. niger*, *F. chlamydosporum*, *F. incarnatum-equiseti* species complex, *F. oxysporum*, *F. subglutinans*, *F. verticillioides*, *Mucor circinelloides*, *Rhizopus oryzae* and *Talaromyces flavus*. The occurrence of *A. niger* in the frass suggests that further studies need to be conducted to determine the effect of *A. niger* infection on fumonisin production in maize in South Africa. DNA quantity of fumonisin-producing *Fusarium* spp. was significantly more in frass collected from greenhouse plants inoculated with *F. verticillioides* and infested with *B. fusca* larvae than in frass collected from the uninoculated and infested control, whilst the field trial showed no significant differences in quantity of target DNA in frass from inoculated and uninoculated plants infested with *B. fusca* larvae. This indicates that plants in the field were naturally infected with *F. verticillioides*.

This study showed that *Bt* maize had no effect on infection of maize ears by fumonisin-producing *Fusarium* spp. and the subsequent production of fumonisin in *F. verticillioides*-inoculated maize ears, indicating that the effect of *Bt* maize on fumonisin production in maize ears is indirectly associated with its control of severe stem borer damage. *Busseola fusca* frass was a reservoir of different fungal species; some pathogenic to maize, and others antagonistic to maize pathogens. Moreover, *B. fusca* infestation of maize stems was associated with higher levels of fumonisin-producing *Fusarium* spp. in larval frass when *F. verticillioides* was present on the plant. Multiple large wounds created by cork borers resulted in significantly more FER symptoms and fumonisin production, irrespective of artificial *F. verticillioides* inoculation of maize ears whereas *B. fusca* infestation resulted in a significant increase in FER in only one of the three seasons, moreover, it had no effect on fumonisin production in all three seasons. This indicates that severe wounds that opens up husk

coverage and exposes maize kernels; caused by factors such as insects, hail and bird damage, and damage by implements; are important entry points for *F. verticillioides* that may lead to the transition from symptomless infection to necrotrophic pathogenicity resulting in FER and concomitant fumonisin production in maize kernels. However, climatic conditions are also important in FER and fumonisin production in maize. Moreover, *Acremonium zeae* endophytes occurring in frass can be used for the biological control of *F. verticillioides* resulting in the management of FER and subsequent fumonisin production.

OPSOMMING

Mielies is ‘n gewas van groot ekonomiese belang in suidelike Afrika wat wyd as stapel voedsel en veevoer dien. Mielieproduksie word egter belemmer deur onderskeidelik patogene en peste soos *Fusarium verticillioides* en die Afrika stamboorder, *Busseola fusca*. *Fusarium verticillioides* infeksie lei tot Fusarium kopvrot (FKV) en die produksie van fumonisien mikotoksiene, terwyl die Afrika stamboorder, *Busseola fusca,* beduidende skade verrig aan mielieweefsel tydens larf voeding. Ondanks pogings om *F. verticillioides* te beheer, bly infeksie en fumonisienproduksie ‘n bedreiging vir mielieproduksie weens die tekort aan weerstandbiedende mieliekultivars en die onvermoë om die patogeen te teiken met swamdoders en biologiese beheerprodukte. Die aanplanting van *Bt* mieliebasters is ’n belangrike meganisme vir die bestuur van mieliestamboorders. Die onlangse ontdekking van *B. fusca* weerstand teen *Bt* mielies met ‘n enkele kristal proteïen geen, dui egter daarop dat daar nie slegs op hierdie tegnologie gesteun moet word vir die bestuur van *B. fusca* nie.

Die interaktiewe effek van *B. fusca* en *F. verticillioides* op FKV en fumonisienproduksie in mielies is tydens hierdie studie ondersoek. Mieliekoppe is slegs *F. verticillioides* geïnokuleer, met beide *F. verticillioides* en *B. fusca*, en met slegs *B. fusca*. *Fusarium verticillioides* isolaat MRC826 is geïnokuleer deur ‘n spoorsuspensie van die fungus in te spuit in die sy kanaal van elke primêre kop tydens die blaas fase. Vir *B. fusca* infestasie, is afmetings van 10-15 neonaat larwe gedeponeer in die krans van elke plant by die 12-13de blaarfase voor pluimvorming, deur gebruik te maak van ‘n meganiese toediener. Mieliekoppe is ook meganies gewond tydens die blaasfase met ‘n kurkboorder (verskillende groottes en hoeveelhede wonde), om sodoende haelskade na te boots, en die helfde van die wonde is geïnfekteer met *F. verticillioides*. Resultate van hierdie studie het aangedui dat die impak van *B. fusca* infestasie op FKV seisoenaal variasie getoon het, moontlik weens die sporadiese skade aan mieliekoppe. *Busseola fusca* infestasie het egter nie gelei tot ‘n beduidende toename in fumonisienproduksie nie. Die graad van verwonding van die mieliekoppe was ‘n belangrike bydraer tot FKV ontwikkeling en fumonisienproduksie.

Die effek van gasheer plant genetiese verandering en plaagdoder toediening op FKV en fumonisienproduksie in mielies is ondersoek deur die reaksie te bestudeer van ‘n *Bt* kruising en sy nie-*Bt* iso-kruising tot *F. verticillioides* infeksie en *B. fusca* infestasie; en deur die behandeling van plante met Beta-cyfluthrin (nie-sistemies) en Benfuracarb (sistemies) insekdoders. Die veldproewe is oor ‘n tydperk van drie seisoene uitgevoer met die gebruik van ‘n ewekansige volledige blokontwerp met ses herhalings per behandeling. Ongeïnokuleerde, nie-geïnfesteerde en onbeskadigde kontrole behandelings is ingesluit. Alle koppe is geoes by fisiologiese volwassenheid en FKV, totale fumonisien konsentrasie,

stamboorder kumulative tonnel lengte (*B. fusca* skade) en die hoeveelheid teiken DNA van fumonisien-produserende *Fusarium* spp. is gekwantifiseer. *Busseola fusca* infestasie het geen effek gehad op swam kolonisasaie en fumonisienproduksie in mielies nie. *Bt* en nie-*Bt* pitte is eweveel gekontamineer met swam DNA, maar FKV en fumonisienproduksie het afgeneem in die *Bt* kruising. Ten spyte van die bewyse gevind in hierdie en ander studies dat *Bt* mielies indirek FKV en fumonisienproduksie verminder, was dit ook strydig oor seisoene. Benfurakarb het stamboorders beheer, en saam met dit ook indirek FKV en fumonisienproduksie verminder. FKV ontwikkeling en fumonisienproduksie deur *F. verticillioides* het oor seisoene gevarieer, wat dui op die belangrikheid van omgewingstoestande vir FKV en fumonisienproduksie.

'n Opname is ook by twee areas in die Noordwes Provinsie en een area in die Vrystaat Provinsie van Suid-Afrika uitgevoer, om mikoflora in *B. fusca* wurmboorsel te analiseer. Blootstelling van *B. fusca* larwe teenoor *F. verticillioides* in staboorder wurmboorsel is ook geëvalueer in beide kweekhuis- en veldproewe. Mieliekranse is met 'n spoorsuspensie van *F. verticillioides* MRC 826 geïnokuleer 4 weke na opkoms en 2 dae later geïnfesteer met afmetings van 5-10 neonaat *B. fusca* larwe. Die kontrole behandeling het bestaan uit slegs *B. fusca* infestasie. Verskeie swamspesies is geassosieer met stamboorder wurmboorsel, insluitend *Acremonium zeae, Aspergillus flavus, A. niger, F. chlamydosporum, F. incarnatum-equiseti* spesiekompleks, *F. oxysporum, F. subglutinans, F. verticillioides, Mucor circinelloides, Rhizopus oryzae* en *Talaromyces flavus*. Die voorkoms van *A. niger* in die wurmboorsel dui daarop dat verdere studies uitgevoer moet word om die effek van *A. niger* infeksie op fumonisienproduksie in mielies in Suid-Afrika te bepaal.

DNA hoeveelheid van fumonisien-produserende *Fusarium* spp. was betekenisvol hoër in wurmboorsel wat versamel is van kweekhuisplante geïnokuleer met *F. verticillioides* en geïnfesteer met *B. fusca* larwe as in wurmboorsel versamel vanaf die ongeïnokuleerde kontrole terwyl die veldproef geen betekenisvolle verskille in kwantiteit teiken DNA in wurmboorsel vanaf geïnokuleerde en nie-geïnokuleerde plante geïnfesteer met *B. fusca* larwe getoon het nie. Dit dui daarop dat plante in die veld natuurlik deur *F. verticillioides* geïnfekteer is.

Die studie het aangedui dat *Bt* mielies geen effek op infeksie van mieliekoppe het by fumonisien produserende *Fusarium* spp. en vervolgens fumonisienproduksie in geïnokuleerde *F. verticillioides* mieliekoppe nie. Dit toon dat die effek van *Bt* mielies op fumonisienproduksie in mieliekoppe indirek met die beheer van stamboorder skade geassosieer is. Hierdie studie het getoon dat *B. fusca* wurmboorsel as reservoir gedien het vir verskeie swamspesies; sommiges patogenies vir mielies, en ander antagonisties tot mieliepatogene. Met infestasie van *B. fusca* op mielie stamme word ondervind dat hoër vlakke van fumonisien produserende *Fusarium* spp. in larwe wurmboorsel geassosieer word met teenwooidigheid van *F.*

verticillioides op plante. Verskeie groot wonde veroorsaak deur kurkboorders het gelei tot beduidend meer FKV simptome en fumonisienproduksie, ongeag kunsmatige *F. verticillioides* inokulasie van mieliekoppe, terwyl *B. fusca* infestasie gelei het tot ‘n beduidende toename in FKV in slegs een van die drie seisoene. Verder het dit geen effek gehad op fumonisienproduksie in enige van die drie seisoene nie. Dit dui daarop dat wonde wat dopbedekking oopmaak en pitte blootstel; veroorsaak deur faktore soos insekte, haelskade, voëlskade, en implimentskade; belangrike toegangspunte is vir *F. verticillioides* wat mag lei tot die oorgang van simptoomlose endofitisme na nekrotrofiese patogenisiteit wat lei tot FKV en gepaardgaande fumonisienproduksie in mieliepitte. Klimaatstoestande is egter ook belangrik in FKV en fumonisienproduksie in mielies. Verder, kan *Acremonium zeae* endofiete wat in wurmboorsel voorkom vir die biologiese beheer van *F. verticillioides* gebruik word wat lei tot die bestuur van FKV en gevolglike fumonisienproduksie.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

Prof A. Viljoen (Stellenbosch University), **Prof B.C. Flett** (ARC-GCI, North-West University) and **Prof J. Van den Berg** (North-West University), my supervisors who provided invaluable support and guidance;

Prof G.P. Munkvold (Iowa State University, USA), for assistance with drafting the project proposal;

Prof J.B.J. van Rensburg (ARC-GCI), **Dr M. Truter** (ARC-PPRI), **Dr A. Erasmus** (ARC-GCI), **Dr B. Janse Van Rensburg** (ARC-GCI), **Dr A. Schoeman** (ARC-GCI), **Ms D. Biya** (ARC-GCI), **Mr O. Rhode** (ARC-GCI) and **Dr J du Toit** (ARC-GCI) for assistance with *Busseola fusca* rearing and supply, phylogenetic analysis, editing and statistical analyses;

Current and former personnel of the ARC-Grain Crops Institute, for technical assistance:

Laboratory: Ms M. Kwele, Dr B. Janse van Rensburg, Ms D. Biya, Ms M. Mahlobo, Ms S. Phokane, Mr A. Tantasi, Mr J. Viviers

Field work: Mr F. Mashinini, Mr J. Bass, Ms Y. Maila, Ms M. Du Toit, Ms S. Nthangeni, Ms K. Makokoe

Research articles: Ms J. Ramaswe, Ms J. Kilian;

The Agricultural Research Council, The Maize Trust and the National Research Foundation of South Africa, for financial support;

My wife Shushu, and my daughter Lachelle, and many others whom I have been in contact with during my PhD studies, including my friends at **Prof Dr Hans-Ulrich Humpf's** laboratory, Institut für Lebensmittelchemie, Universität Münster, Germany.

CONTENTS

CHAPTER 1

The *Fusarium verticillioides* x *Busseola fusca* interactions in maize: A review

INTRODUCTION

Maize (*Zea mays* L.) originated in Mexico about 7 000 years ago (Ranum *et al*., 2014). Today the crop is produced throughout the world, with the United States, China and Brazil among the top three maize-producing countries, with approximately 65% of the total worldwide production in 2014/15 (USDA, 2016). South Africa is in the top ten maize-producing countries and was top-ranked in Africa in 2014/15 (USDA, 2016). In developed countries maize is processed into food, feed and industrial products that include starch, sweeteners, oil, beverages, glue, industrial alcohol and fuel ethanol. Approximately 40% of maize produced in the United States is now used for ethanol production (Ranum *et al*., 2014).

Maize is the staple food for many people in southern Africa and is a major constituent of animal feeds (SAGIS, 2014). In South Africa the crop is commercially cultivated in eight of the nine provinces, including the Free State, Mpumalanga, North West, Gauteng, KwaZulu-Natal, Limpopo and the Eastern and Northern Cape provinces. The bulk of the commercial maize production occurs within the Maize Triangle (De Waele and Jordaan, 1988), comprising of the western Free State, North West, Gauteng and western Mpumalanga provinces. Annual maize production by commercial farmers for the 2013/14 growing season was 14.3 million tonnes from 2.7 million hectares (ha) of land. Non-commercial maize production amounted to 675 000 tonnes from 408 000 ha (DAFF, 2014; SAGIS, 2014). Dry land farmers produced an average of 3.58 tons/ha while irrigation farmers produced an average of 10 tons/ha in the 2013/14 season (SAGIS, 2014).

One of the most damaging pathogens of maize is *Fusarium verticillioides* Sacc. Nirenberg (syn = *F. moniliforme* Sheldon), a fungus that is frequently associated with the crop in most production areas of the world (Desjardins, 2006; Ncube *et al.*, 2011). *Fusarium verticillioides* is best known for causing Fusarium ear rot (FER), but the symptoms it cause can vary from non-symptomatic infections to severe rotting of roots, stems and ears (White, 1999). The most detrimental effect of *F. verticillioides*, however, is that it produces fumonisin mycotoxins that have been associated with diseases of humans and livestock (Marasas, 2001). *Fusarium verticillioides* is also a pathogen of rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* L.) and sugarcane (*Saccharum officinarum* L.) (McFarlane and Rutherford, 2005; Leslie and Summerell, 2006; McFarlane *et al*., 2009).

Maize production is also negatively affected by stem borer infestations (Porter *et al*., 1991; Kfir, *et al*., 2002; Hutchison *et al*., 2010). The African stem borer, *Busseola fusca* Fuller (Lepidoptera: Noctuidae), is indigenous to sub-Saharan Africa and causes damage to all plant parts in cultivated crops (Calatayud *et al*., 2014). In South Africa the insect is prevalent in the Highveld and the western maize production areas of the country (Kfir and Bell, 1993; Kfir, 2000; 2002), and can cause an estimated annual loss of 10-60% of total maize production (Kfir *et al*., 2002). *Busseola fusca* occurs under cool and wet conditions at altitudes ranging from sea level to 2 000 m above sea level (Abate *et al*., 2000), and is the most injurious pest of maize in South Africa (Annecke and Moran, 1982; Van Rensburg *et al*., 1988a). *Busseola fusca* also causes damage to sorghum (Kfir *et al*., 2002), pearl millet (*Pennisetum glaucum* L.) (Harris and Nwanze, 1992) and sugarcane (Assefa *et al*., 2015).

Planting of genetically modified maize hybrids (*Bt* maize) with *Bacillus thuringiensis* Berliner 1915 genes encoding for the δ-endotoxin crystal proteins that are toxic to lepidopteran insects has become an important method for the management of stem borers (Hellmich *et al*., 2008). However, most subsistence farmers in South Africa and all farmers in countries where restrictions on planting *Bt* maize are in place (Meissle *et al*., 2010), still rely on traditional pest management methods such as insecticides and residue management to control maize lepidopteran pests (Ncube, 2008). The interaction between *B. fusca* and *F. verticillioides*, however, is not sufficiently understood. This review, therefore, summarises available literature on the effect of stem borers such as *B. fusca* and *F. verticillioides* on FER and fumonisin production in maize.

THE FUSARIUM EAR ROT PATHOGEN: *FUSARIUM VERTICILLIOIDES*

Fusarium verticillioides was previously known as *F. moniliforme*, but its name has been changed by Seifert *et al*. (2003) based on the fact that *F. moniliforme* represented an unacceptably broad species concept. *Fusarium verticillioides* was undisputedly the older name for the maize pathogen. *Fusarium verticillioides* has a sexual stage, which was previously known as *Gibberella moniliformis* Wineland (Leslie and Summerell, 2006). *Fusarium verticillioides* is easily recognised by its cultural and morphological characteristics. It produces white mycelia that turn violet when grown on potato dextrose agar. On carnation leaf agar it forms macroconidia that are relatively long and slender, as well as microconidia that are produced in long chains on monophialides (Leslie and Summerell, 2006). During the sexual stage, ascospores are produced in perithecia (Leslie and Summerell, 2006).

Ear rot diseases of maize can be produced by other *Fusarium* spp. too. These species include *F. proliferatum* Matsushima, Nirenberg; *F. boothii* O'Donnell, Aoki, Kistler & Geiser; *F. equiseti* (Corda) Sacc; *F. graminearum* Schwabe; *F. oxysporum* Schlechtend, Emend, Snyder

& Hansen; *F. semitectum* Berk. & Ravenel; *F. subglutinans* Wollenw. & Reinking, Nelson, Toussoun & Marasas (Summerell *et al*., 2003) and *F. temperatum* Scauflaire & Munaut (Schoeman, 2014; Zhang *et al*., 2014). Of these *F. proliferatum, F. temperatum* and *F. subglutinans* cause FER, whereas *F. graminearum* and *F. boothii* are responsible for Gibberella ear rot. Gibberella ear rot reduces maize yields and quality, and results in the contamination of maize ears with zearalenone and trichothecene mycotoxins (Desjardins, 2006). Other ear rots that occur in maize include Aspergillus ear rot and Diplodia ear rot. Aspergillus ear rot is caused by *Aspergillus flavus* Link ex Fr and *Aspergillus parasiticus* Speare (Gourama and Bullerman, 1995), which produce aflatoxin mycotoxins in maize (Diener *et al*., 1987). Diplodia ear rot is caused by *Stenocarpella maydis* (Berk.), which results in diplodiosis, a nervous disorder in cattle (*Bos taurus* L.) and sheep (*Ovis aries* L.) (Snyman *et al*., 2011).

Life cycle of *F. verticillioides*

Kernel infection of maize is mainly due to airborne spores of *F. verticillioides* that infect through the silk channel (Munkvold and Carlton, 1997; Galperin *et al*., 2003) during the silking growth stage of the maize plant (Fig. 1). Airborne spores are produced as microconidia on the previous crop residue, and are disseminated by wind (Munkvold and Desjardins, 1997). After landing on the silks, the fungus progresses through the silk channel and into maize kernels. FER symptoms develop on ear tips or randomly as scattered areas of infection over the ear surface (Parsons and Munkvold, 2012). Direct invasion of kernels may also occur through stress cracks in the pericarp and through the pedicel (Odvody *et al*., 1997; Parsons and Munkvold, 2012), or by means of wounds caused by insects (Munkvold *et al*., 1997) and hail (Robertson *et al*., 2011). At the end of the maize-growing season, the fungus survives in maize residues as thickened hyphae on moist soils (Fig. 2) (Cotten and Munkvold, 1998; Munkvold, 2003). It does not produce chlamydospores, and thus cannot survive for extended periods (Leslie and Summerell, 2006).

Fusarium verticillioides that survives on maize residues at the end of a planting season is sometimes released back into the soil (Munkvold and Desjardins, 1997). At the onset of a new maize-growing season, the soilborne hyphae germinate and grows into lateral roots (Oren *et al*., 2003), after which the fungus colonises the stems of seedlings. Seeds, when contaminated with *F. verticillioides* in the field, can also result in systemic infections of maize seedlings. This growth of the fungus may continue asymptomatically until seed production, when the fungus grows into the ear and colonise maize kernels (Fig. 2).

Pathogenicity of *F. verticillioides*

Fusarium verticillioides causes damping-off, stem malformation, as well as ear, stem and root rot of maize (Nelson *et al*., 1993; Leslie and Summerell, 2006). Of these, FER is considered the most important. FER becomes visible as pink or white fungal growth on damaged and undamaged kernels (White, 1999). Some kernels do not become discoloured, but rather show white streaks with a starburst appearance (Duncan and Howard, 2010). *Fusarium verticillioides* can also infect maize kernels without showing any visible symptoms (Oren *et al*., 2003). Plant malformation manifests as twisted foliage and tillers (Cardwell *et al*., 2000). Stem rot results in wilted plants with greyish-green leaves (White, 1999), of which the internal pith of the lower stem becomes soft and disintegrated. Mycelial growth can be observed at lower nodes (White, 1999), and the stem exhibits a reddish discolouration internally when split open (White, 1999). Fusarium root rot symptoms appear as brownish lesions on the roots, and develop as early as the 6th leaf stage on the primary roots. When root rot continues to develop it can become severe on the adventitious roots (Sparks, 2016). This may reduce the length of primary roots and the quantity of secondary roots (Soonthornpoct *et al*., 2000).

Fusarium verticillioides is both an endophyte and a pathogen of maize (Yates *et al*., 1997). The transition from endophytic stage to the pathogenicity stage is initiated by the single orthologous gene, *SGE1*, which regulates markedly different genes in different fungi (Brown *et al*., 2014). In *F. verticillioides*, *SGE1* regulates secondary metabolism and pathogenicity to maize (Brown *et al*., 2014). Pathogenicity of *F. verticillioides* is also regulated by other genes such as the *FvSO* gene, which is required for vegetative growth and sporulation, fumonisin production and pathogenicity in *F. verticillioides* (Guo *et al*., 2015). Boudreau *et al*. (2013) showed that mutant *F. verticillioides* strains lacking a *TPS1* gene, which encodes a putative trehalose-6-phosphate synthase, produced significantly less fumonisin and were less pathogenic than the non-mutant strain to maize. Moreover, Zhang *et al*. (2011) indicated that a mutant *F. verticillioides* strain lacking the *FvMK1* gene, that regulates conidiation, pathogenesis, and fumonisin production, was non-pathogenic and did not colonise through wounding sites and failed to cause stem rot symptoms beyond the inoculation sites on maize stems. The *F. verticillioides* velvet gene *FvVE1* also regulates stem rot symptom production and fumonisin synthesis in maize seedlings (Myung *et al*., 2012). Maize plants, grown from seeds inoculated with the *FvVE1* deletion mutant, did not develop disease symptoms while plants grown from seeds inoculated with the *F. verticillioides* non-mutant strain developed disease symptoms (Myung *et al*., 2012).

Toxigenicity of *F. verticillioides*

Fumonisins in maize are produced by *F. verticillioides*, *F. proliferatum* (Nelson *et al*., 1983; Leslie and Summerell, 2006) and *Aspergillus niger* (Tiegh.) (Frisvad *et al*., 2011). Of these, *F.*

verticillioides is considered the most important producer of fumonisins worldwide (Desjardins, 2006; Picot *et al*., 2010). The mycotoxin was first isolated from maize collected in the Eastern Cape province of South Africa by Gelderblom *et al*. (1988). Since then, a family of fumonisin analogues have been characterised and grouped into fumonisins A, B, C and P series. Fumonisins produced by *F. verticillioides* consist primarily of the B series fumonisins, referred to as FB_1, FB_2 and FB_3. Of these analogues, FB_1 is the most important with a wide geographic distribution worldwide (Thiel *et al*., 1992; Musser and Plattner, 1997; Rheeder *et al*., 2002). Fumonisins can also be found in sorghum kernels (Shetty and Bhat, 1997), grapes (*Vitis vinifera* L.) and raisins (Mogensen *et al*., 2010).

FB_1 has been classified as a group 2B carcinogen by the International Agency for Research on Cancer (IARC), indicating that it is a possible carcinogen to humans (IARC, 1993). It inhibits the synthesis of sphingolipids (He *et al*., 2001; Riley *et al*., 2006), thereby interfering with the function of membrane proteins resulting in neural tube defects (NTD). Potential mechanisms for fumonisin hepatotoxicity and carcinogenicity include fatty acid accumulation and cell proliferation, oxidative stress, lipid peroxidation, peroxisome proliferation and disruption of the production of cellular lipids (Riley *et al*., 2006). Contamination of maize with FB_1 has been correlated with the occurrence of human oesophageal cancer in the Eastern Cape province of South Africa (Rheeder *et al*., 1993) and the Cixian, Linxian and Shangqiu counties of China (Chu and Li, 1994; Yoshizawa e*t al*., 1994), and a high incidence of NTD in infants whose mothers were consuming fumonisin-contaminated maize during pregnancy in Mexico (Missmer *et al*., 2006). However, it is known that maternal folic acid protects the foetus against NTD, and fortification of all enriched cereal products with folic acid reduces the occurrence of NTD (Green, 2002).

Exposure of animals to FB_1 in feed results in a number of clinical symptoms. The most dramatic manifestation of maize feed contaminated with fumonisin is equine leukoencephalomalacia (ELEM), a neurotoxic disease of horses (*Equus ferus caballus* L.) and donkeys (*Equus africanus asinus* L.) that is induced by FB_1 (Kellerman *et al*., 1990; Jovanović *et al*., 2015). Contaminated feed containing FB_1 levels as low as 8 µg/g FB_1 exposes ponies to an elevated risk of ELEM development (Wilson *et al*., 1992; Marasas, 1995). FB_1 also induces porcine pulmonary oedema in pigs (*Sus scrofa domestica* L.) (Kriek *et al*., 1981; Harrison *et al*., 1990) and hepatocellular carcinoma, cholangiofibrosis and cholangiocarcinoma in rats (*Rattus spp.* Fischer de Waldheim) (Gelderblom *et al*., 1991). A number of species-specific effects have been experimentally induced by fumonisins on other animals such as immuno-suppression in chickens (*Gullus domesticus* L.), toxicity to broiler chicks and chicken embryos and nephrotoxicity in rabbits (*Oryctolagus cuniculus* L.) (Marasas, 1995).

Surveys conducted in Africa, North America, South America, Europe, the Middle East, South Asia, East Asia, Australia and New Zealand have shown that fumonisins occur in maize worldwide (Desjardins, 2006). The maximum tolerable daily intake for fumonisins recommended by the Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) is 2 µg/g fumonisin per kg of human body weight per day in humans (WHO, 2002). The US Food and Drug Administration maximum levels for fumonisins in human food are set at 2 µg/g (FDA, 2001), while the European Union has maximum levels of 1 µg/g (EC, 2007) in food intended for direct human consumption. In South Africa, the maximum allowable limit for fumonisins in human food has recently been legislated at 2 µg/g (DOH, 2016). The maximum allowable limit in animal feed is, however, legislated in many countries (FAO, 2003).

Relationship between pathogenicity and toxigenicity

The occurrence of FER and the production of fumonisins are distinct aspects of *F. verticillioides* infection in maize. Fumonisins have been shown to occur in maize ears without visible FER symptoms (Munkvold *et al*., 1997; Sobek and Munkvold, 1999). Desjardins and Plattner (2000) indicated that fumonisin production is not required by *F. verticillioides* for maize ear infection and the development of FER. Glenn *et al*. (2008), however, demonstrated that *F. verticillioides* from banana (*Musa acuminata* Colla); which neither produced fumonisins nor caused disease to maize seedlings; became pathogenic and produced fumonisin on maize seedlings after the banana strain was transformed with the fumonisin biosynthetic gene (*FUM*) cluster. This pathogenesis was only demonstrated for foliar diseases on maize seedlings (Glenn *et al*., 2008).

Factors modulating Fusarium ear rot and fumonisin production in maize

Environmental conditions

Fusarium verticillioides and fumonisin production are predominant in maize grown in warmer and drier climates, as well as cooler and moist climates (Ngoko *et al*., 2001; 2002; Santiago *et al*., 2015). A model with non-linear, 3-dimensional Lorentzian equation indicated that fumonisin production is largely influenced by climatic conditions such as temperature, rather than by rainfall, during the dough stage of kernel fill (Janse van Rensburg, 2012). Temperatures exceeding 35°C induce plant stress in maize and promote systemic infection of maize by *F. verticillioides* (Murillo-Williams and Munkvold, 2008). Decreasing soil moisture, and high soil and air temperatures during the later stages of ear maturity and dry down exacerbates silk cut, which causes lateral splits in the kernel pericarp (Murillo-Williams and Munkvold, 2008), thereby exposing the kernel tissues and embryo to *F. verticillioides* infection (Odvody *et al*., 1997). When grown outside their range of adaptation, hybrids appear to be

more susceptible to *F. verticillioides* infection and concomitant fumonisin production (Shelby *et al*., 1994; Miller, 2001). However, hybrid x season and season x location interactions are significant sources of variation for FER and fumonisin production, respectively (Venturini *et al*., 2015).

Physio-chemical composition of kernels

Starch formation during kernel ripening progressively reduces water activity (a_w) to levels that may trigger fumonisin production by *F. verticillioides* (Picot *et al*., 2010). The highest levels of FB_1 production occur at the dent stage and the lowest at the blister stage (Fig. 1) (Warfield and Gilchrist, 1999). The pH and carbon:nitrogen ratio fluctuations during the course of maize ripening also modulate fumonisin production (Picot *et al*., 2010) and fumonisin biosynthesis in *F. verticillioides* is repressed by high nitrogen levels and alkaline pH (Flaherty *et al*., 2003). However, pH levels above 3.5 were found to enhance fungal growth in liquid cultures (Keller *et al*., 1997). The optimum production of fumonisins occurs under relatively high oxygen tensions and low pH due to organic acids produced by starch metabolism of *F. verticillioides*-colonised maize kernels (Miller, 2001).

The chemical composition of the pericarp in maize kernels plays a role in FER development and fumonisin production (Santiago *et al*., 2015). High ferulic acid content in the pericarp and aleurone tissues increases maize resistance to fungal infection (Bily *et al*., 2003). High levels of phenylpropanoids; such as *trans*- and *cis*-ferulic acid, *p*-coumaric acid and diferulate esters; have been shown to be associated with reduced FER and fumonisin production (Sampietro *et al*., 2013) due to their inhibitory effect on *F. verticillioides* (Santiago *et al*., 2015). Studies by Venturini *et al*. (2015) indicated that flavonoids are an important component in the resistance of maize to FER and fumonisin production. Significant differences between two isogenic hybrids (isohybrids), one with pigmentation in the pericarp and the other without pigmentation, were observed, with the pigmented kernels less affected by FER and fumonisin production than non-pigmented kernels.

Insects

Wounds produced by insects provide sites for infection of maize ears and stems by airborne or rain-splashed inoculum of *F. verticillioides* (Munkvold and Desjardins, 1997). Kernel damage caused by lepidopteran stem borers, such as the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae), has been associated with high FER development and fumonisin production in the USA (Munkvold *et al*., 1997). In South Africa, maize stem borers, mainly *B. fusca*, were also associated with a higher incidence of FER in maize (Flett and Van Rensburg, 1992).

Production practices

Production practices such as tillage, crop rotation, planting date, planting density and fertiliser treatment can influence FER and fumonisin production in maize. FER pathogens such as *F. verticillioides, F. proliferatum* and *F. subglutinans* survive longer in fields with surface maize residue when compared to fields with buried residues (Cotten and Munkvold, 1998). The primary inoculum in such residues can become airborne to infect maize ears and colonise kernels (Oren *et al*., 2003). Conservation tillage systems have been shown to sustain a higher diversity of *Fusarium* spp. than moldboard plough-based tillage systems (Steinkellner and Langer, 2004). No-till farming of maize following oats (*Avena sativa* L.) in Brazil resulted in higher fumonisin levels than maize conventional tillage following oats (Ono *et al*., 2011).

Late planting of maize, maize monoculture and crop rotation with other cereal crops in the same or in adjacent fields increase the build-up of *F. verticillioides* inoculum (Dowd, 2003). Late season planting affects kernel integrity, and thereby result in a significant increase in FER and FB_1 production. In contrast, early planting consistently resulted in lower FER and FB_1 levels in California and Hawaii compared to late planting dates (Parsons and Munkvold, 2012). High plant densities have increased maize kernel infection with *F. verticillioides* coupled with more FER compared to fields with lower plant densities (Blandino *et al*., 2008a). Plant densities that exceed agronomically recommended levels increase demand for water and nutrients, and such competition results in plant stresses that predispose plants to fungal infection and concomitant mycotoxin production (Bruns, 2003). Nitrogen deficiency caused increased fumonisin production in maize in Italy (Blandino *et al*., 2008b), and fumonisin levels have been shown to decrease with an increase in nitrogen fertilization rates (Ono *et al*., 2011).

Management of Fusarium ear rot and fumonisins

Biological methods

Several bacterial and fungal species suppress *F. verticillioides in vitro* and *in planta*. The bacterial maize endophyte *Bacillus mojavensis* releases Leu-7 surfactants suppress *F. verticillioides in vitro* (Bacon and Hinton, 2002; Snook *et al*., 2009; Bacon and Hinton, 2011) by forming micelles that result in high membrane-destabilising activity and solubilisation of the fungal membranes (Heerklotz and Seelig, 2001). As a seed dressing, however, its effectiveness was limited. It is believed that the production of fusaric acid by *F. verticillioides* prevents *B. mojavensis* from protecting seedlings against the fungus (Bacon *et al*., 2004; 2006). *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* reduced *F. verticillioides* and fumonisin accumulation in maize kernels when applied as seed dressings at a concentration of 10^7 colony forming units ml^{-1} (Pereira *et al*., 2007). Seed dressing with *Bacillus cereus sensu lato* has also reduced the incidence of FER and fumonisin production in maize in Mexico (Lizárraga-Sánchez *et al*., 2015). Nayaka *et al*. (2009) further showed that

Pseudomonas fluorescens effectively reduced the incidence of *F. verticillioides* and the level of fumonisins in maize when used as a seed and spray treatment.

Acremonium zeae Gams & Sumner inhibited the growth of *F. verticillioides* in cultural tests (Wicklow *et al*., 2005). This inhibition is due to the production of antibiotics, known as pyrrocidines A and B (Wicklow *et al*., 2005), which inhibit protein biosynthesis (Menninger, 1995). *Trichoderma harzianum* seed treatment reduced *F. verticillioides* infection by 58% and fumonisin production by 53% in maize in Italy (Ferrigo *et al*., 2014b), and can be used as an environmentally friendly method to reduce *F. verticillioides* infection (Sobowale *et al*., 2005; 2007). Root colonisation of maize by *T. harzianum* consistently reduced the pathogenicity of *F. verticillioides* by activating the jasmonic acid (JA), salicylic acid (SA) and ethylene (ET)-dependent defence mechanisms in maize plants (Ferrigo *et al*., 2014a).

Chemical methods

A number of fungicides were evaluated against *F. verticillioides* in the laboratory and in the field. Tebuconazole reduced the growth of *F. verticillioides* and *F. proliferatum in vitro*, but did not reduce the production of fumonisins (Marín *et al*., 2013). Maize seed treated with a protectant consisting of 35% Thriophate-methyl, 20% Thiram and 15% Diazinon, combined with an 80% Benomyl solution sprayed directly on the soil, tended to have similar *F. verticillioides* stem infection as the *F. verticillioides*-uninoculated control following artificial infection with *F. verticillioides* on the first internode (Schulthess *et al*., 2002). However, De Curtis *et al*. (2011) demonstrated that soil and foliar application of Tebuconazole and Tetraconazole, as well as the combination of Prochloraz + Cyproconazole with the insecticide Lambda-cyhalothrin, significantly reduced fumonisin production when applied to the soil and maize foliage. Lambda-cyhalothrin consistently reduced insect damage severity when applied alone, while fumonisin production was only reduced in 50% of cases (De Curtis *et al*., 2011).

In Europe, more than 95% of the maize seeds planted are fungicide-treated. The treatments include amide, dithiocarbamate and pyrrole foliar fungicide sprays used against *Fusarium* spp. for seed production (Meissle *et al*., 2010). Chemical elicitors, such as β-amino butyric acid, benzothiadiazole, harpin protein, 2,6-dichloroisonicotinic acid and methyl jasmonate (MeJA), were unable to induce resistance to FER and fumonisin production (Small *et al*., 2012b). The optimisation of elicitor application method, dosage rate and frequency, as well as timing of application, could potentially increase plant response against *F. verticillioides*.

Cultural practices

Fusarium verticillioides infection and the subsequent accumulation of mycotoxins in maize kernels can be controlled through cultural practices such as tillage, crop rotation and the application of fertilizers (Munkvold, 2003; Parsons and Munkvold, 2010). Removal of crop

residues from previous crops also reduced primary inoculum levels in the field (Dragich and Nelson, 2014). The survival of *F. verticillioides* can further be reduced by ploughing-in of surface residues (Cotten and Munkvold, 1998). Steinkellner and Langer (2004) indicated that moldboard ploughing resulted in a lower diversity of *Fusarium* spp. than the chisel plough and rotary tiller treatments, and the deeper the tillage the lower the number of surviving *Fusarium* spp. However, Flett *et al*. (1998) found that tillage practices applied prior to planting; such as rip-on-rowing followed by tiller, moldboard ploughing, shallow chisel, V-blade ploughing and disk-ploughing; had no effect on ear rots caused by *Fusarium* spp.

Crop rotation of maize with non-host crops of *F. verticillioides* breaks the life cycle of the pathogen and reduces FER and concomitant fumonisin production. Crop rotation with beans (*Phaseolus spp.* L.), groundnut (*Arachis hypogaea* L.) and potatoes (*Solanum tuberosum* L.) have all been demonstrated to reduce primary fungal inoculum (Atukwase *et al*., 2009; Dragich and Nelson, 2014). Sub-soiling in compacted soils minimises plant stress (Bruns, 2003) and reduces stress levels of maize in the field. Blandino *et al*. (2008b) also indicated that fumonisin contamination was highest in maize fields with nitrogen deficiencies.

Plant resistance

Progress made in genetic improvement of maize for *F. verticillioides* resistance has been limited. Breeding efforts to increase resistance to FER is uncommon due to the fact that ear rots rarely result in yield losses (Mesterházy *et al*., 2012). A hybrid named Mona was found to be resistant to *F. verticillioides* and *F. proliferatum* in Poland (Pascale *et al*., 2002), while Clements and White (2004), Afolabi *et al*. (2007) and Small *et al*. (2012a) found maize inbred lines in which FER was significantly reduced in the USA, Nigeria and South Africa, respectively in maize ears inoculated with *F. verticillioides*.

Post-harvest storage and treatment

The risk of fumonisin production increases in post-harvest maize kernels with high moisture levels (Warfield and Gilchrist, 1999). A moisture level exceeding 18% is favourable for *F. verticillioides* growth and concomitant fumonisin production during storage (Kommedahl and Windels, 1981). Subsistence farming practices; such as harvesting maize with a high moisture content and storing it in drums and tanks, as well as the non-control of weevils (*Sitophilus zeamais* Motschulsky) and other insects (Ncube, 2008); may thus promote fungal growth and mycotoxin production.

Standard storage procedures that prevent production of fumonisins in kernels, such as drying of maize kernels to moisture levels below 18% within 1-2 days after harvest, are recommended. Stored kernels should be aerated to reduce moisture content, and temperature set to 20˚C (Joao and Lovato, 1999). Adjusting the combine harvester to avoid kernel damage

during harvesting further reduces fungal infection that could lead to mycotoxin production (Munkvold and Desjardins, 1997; Bruns, 2003). Bennett and Richard (1996) found that the gluten and fibre fractions of fumonisin-contaminated maize extracted by wet milling, rather than the starch, contained considerable amounts of fumonisins. These fractions, thus, require further decontamination before being used in animal feed. For subsistence farming systems, hand sorting mouldy from non-mouldy maize kernels prior to consumption is recommended to reduce fumonisin exposure (Desjardins *et al*., 2000; Van der Westhuizen *et al.*, 2010).

Specific processes used in food preparation; such as nixtamalisation of maize dough (Palencia *et al*., 2003), the use of adsorbent clays (Baglieri *et al*., 2013), and heat treatment (Jackson *et al*., 1996); can reduce fumonisin levels in maize. The nixtamalisation of maize dough, however, may result in hydrolysed fumonisins that can be nearly as toxic as the unaltered FB_1 (Murphy *et al*., 1996). Lu *et al*. (1997) found that non-enzymatic browning that occurs in the presence of a primary amine, a reducing sugar, and water at pH above 7, resulted in the removal of the primary amine group from the fumonisin molecule and concomitant reduction in detectable FB_1 levels. Treatment of contaminated maize kernels with a combination of hydrogen peroxide and sodium bicarbonate reduced end-product toxicity (Park *et al*., 1996). Reduction in the bioavailability of fumonisins can also be achieved through the use of adsorbent clays, activated carbons and cholestyramine that have a high affinity for FB_1. These compounds all tightly bind and immobilise FB_1 in the gastro-intestinal tract of livestock and the immobilised FB_1 is then excreted (Huwig *et al*., 2001; Solfrizzo *et al*., 2001). Cholestyramine has the best FB_1 adsorption capacity, followed by activated carbon, bentonite and celite in rats (Solfrizzo *et al*., 2001). Fumonisins are fairly heat stable, but can be reduced by 16-100% when moist or dry maize kernels are heated at temperatures exceeding 160˚C for 20-60 min (Jackson *et al*., 1997; Castelo *et al*., 1998; Katta *et al*., 1999).

THE AFRICAN MAIZE BORER *BUSSEOLA FUSCA*

Busseola fusca is the most important pest of maize in South Africa (Kfir and Bell, 1993; Kfir, 2000; 2002) while the spotted stem borer, *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae), is of lesser economic importance (Kfir, 2002). The African pink stem borer, *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae), is also considered a minor pest of maize in the country (Van den Berg, 1997; Van den Berg and Drinkwater, 2000). Some other Lepidoptera spp. that have attained pest status in South Africa include the black cutworm (*Agrotis ipsilon* Hufnagel), brown cutworm (*Agrotis longidentifera* Hampson), grey cutworm (*Agrotis subalba* Walker), and common cutworm (*Agrotis segetum* Denis & Schiffermüller), African bollworm (*Helicoverpa*

armigera Hübner), army worm (*Spodoptera exempta* Walker), and beet army worm (*Spodoptera exigua* Hübner) (Annecke and Moran, 1982).

Life cycle of *B. fusca*

Pupal and moth stage

The life cycle of *B. fusca* consists of the adult female moth laying eggs that hatch into larvae and the larvae turning into pupae, from which adult moths will emerge to complete the cycle (Fig. 3). In South Africa *B. fusca* produces neither two nor three generations per season, but a combination of these (Mally, 1920; J.B.J. van Rensburg, personal communication). Three generations may occur if moths emerge early in spring (August). However, when a third generation of moths appear in the following spring, only two generations occur in that year. Pupae of the first summer generation occur from the end of December to January, and adults emerge to oviposit from the end of January to the first week in February on maturing plants (Mally, 1920). The larvae matures during March and April and some of these larvae pupate and emerge as moths, giving rise to a partial third generation. The larvae of these and the remainder of the second generation go into diapause in maize stubble (Mally, 1920). Adult moths mostly emerge at night and females release pheromones soon after emergence to attract males for mating (Harris and Nwanze, 1992). After mating female moths disperse in search of suitable host plants for oviposition over 3-4 successive nights (Harris and Nwanze, 1992).

Egg and larval stage

Busseola fusca moths oviposit their eggs on maize during the early vegetative stages. Oviposition after tasselling occurs only when younger plants are not readily available (Van Rensburg *et al*., 1987b). The range of plant ages during which oviposition normally occurs might be extended in slow-growing hybrids and, therefore, slow-growing hybrids are prone to higher levels of infestation (Van Rensburg *et al*., 1989). Moths oviposit eggs in batches of 30-100 on the inner surfaces of leaf sheaths or on other smooth surfaces, such as the plant stem, during the early vegetative stages (Harris and Nwanze, 1992). The eggs are white initially, and darken with age, until they hatch into larvae in about 9 days (Bijlmakers, 1989; Harris and Nwanze, 1992; Robertson, 2000). The larvae then migrate to the whorl where feeding on the leaves takes place. Neonate larvae disperse within and between plants via 'ballooning-off', whereby first instar larvae cling onto silk threads enabling wind-facilitated movement (Kaufmann, 1983; Zalucki *et al*., 2002) during the pre-feeding movement phase (Zalucki *et al*., 2002; Calatayud *et al*., 2015). In addition to full ballooning, Calatayud *et al*. (2015) proposed that *B. fusca* neonate larvae migrate between plants by actively crawling, their orientation

toward host plants being guided by host plant-secreted volatiles. However, this form of larval migration has not yet been demonstrated under field conditions (Calatayud *et al*., 2015).

The majority of larvae pass through winter (diapause) in the base of the stem where they are sheltered from natural predators and adverse climatic conditions (Bijlmakers, 1989; Kfir, 1991; Harris and Nwanze, 1992; Robertson, 2000). Factors such as an increase in carbohydrate and decrease in protein and water are responsible for the induction of the diapause phase in *B. fusca* (Kfir, 1993a; 1993b). The larvae then emerge from diapause and pupate in the following spring between September and November (Kfir, 1991; Harris and Nwanze, 1992). Where maize is cultivated throughout the year, first generation moths that start their development process earlier tend to go through a third generation within the development year (Bijlmakers, 1989; Harris and Nwanze, 1992; Robertson, 2000). The maize crop planted during the summer in South Africa is usually exposed to a single generation of *B. fusca* larvae (J.B.J. Van Rensburg, personal communication).

Busseola fusca **host plants**

Maize and sorghum are the best-known host crops for *B. fusca* (Kfir *et al*., 2002). Recent studies have indicated that the *B. fusca* host range has expanded to include sugarcane in small-scale farming systems in Ethiopia and southern Africa (Assefa *et al*., 2015). Sugarcane is grown adjacent to or in mixed systems with maize in small-scale farming systems, and this practice could have brought *B. fusca* in contact with sugarcane (Assefa *et al*., 2015). Moreover, this may have been facilitated by the presence of sugarcane as the only host during the winter when *B. fusca* is ordinarily in diapause in maize plants (Assefa *et al*., 2010; 2015).

Other *B. fusca* host plants in South Africa include giant reed (*Arundo donax* L.), citronella grass (*Cymbopogon nardus* L.) Rendle and common wild sorghum (*Sorghum arundinaceum* Desv.) Stapf (Calatayud *et al*., 2014). *Busseola fusca* has also been found on Guinea grass (*Panicum maximum* Jacq.), Napier grass (*Pennisetum purpureum* Shumach.), lemon grass (*Cymbopogon giganteus* Chiov.), pearl millet, and bigleaf bristle grass (*Setaria megaphylla* Steud.) Duran & Schinz in East Africa (Calatayud *et al*., 2014). *Busseola phaia* Bowden, *B. segeta* Bowden and *B. nairobica* Le Rü, which closely resemble *B. fusca*, occur on wild host plants around cereal crops. However, *B. fusca* accounts for only about 14% of all the *Busseola* spp. collected on wild hosts (Calatayud, *et al*., 2014).

Behaviour of *B. fusca* moths and larvae on host plants

Oligophagous insects such as *B. fusca* have a narrow host range due to strong selectivity (Bernays and Chapman, 1994). The gravid female moth is primarily responsible for host plant recognition and selection (Van den Berg, 2006). Recognition and colonisation is based on the interaction between the insect's sensory systems and the physicochemical characteristics of

its immediate environment (Udayagiri and Mason, 1995; Calatayud *et al*., 2006). The sensory equipment of the moth consists of multi-porous chemoreceptor and mechanoreceptor sensillae on the antennae which receive plant volatiles, and uniporous gustatory sensilla on the antennae, tarsi and ovipositor that probes the plant surface (Calatayud *et al*., 2006).

Lepidopteran insects use various sensory cues (stimuli) to locate and accept host plants (Bernays and Chapman, 1994). Visual and olfactory cues play an essential role before the moth lands on the plant (Rojas and Wyatt, 1999), whereas olfactory and tactile cues are thought to be more crucial after landing (Hora and Roessingh, 1999). The moth acquires information on the quality and suitability of the plant for colonisation upon landing (Renwick and Chew, 1994). The final behavioural sequence leading to acceptance or rejection of the site for oviposition depends mainly on contact cues consisting of both physical factors such as pubescence and surface texture, and chemical cues such as volatile and surface chemicals present (Hora and Roessingh, 1999).

The host plant selection by insects is usually divided into 'host plant finding' and 'host plant acceptance' (Finch and Collier, 2000). During host plant finding, the searching moths avoid landing on brown surfaces such as soil, but lands indiscriminately on green objects such as leaves of host plants. Landing on green plant tissue is referred to as 'appropriate landings', whereas landing on non-host plants is called 'inappropriate landings' (Finch and Collier, 2000). After landing, the female typically sweeps her ovipositor on the plant surface, simultaneously touching it with the tips of her antennae, and then oviposit (Renwick and Chew, 1994; Calatayud *et al*., 2008). This behaviour is more frequently observed in maize and sorghum, indicating that both antennal and ovipositor receptors are used by the female moths to evaluate the plant surface before ovipositing (Calatayud *et al*., 2008). Females recognise their preferred hosts only after landing where tactile and contact-chemoreception cues from the plants play a major role in oviposition decisions (Calatayud *et al*., 2008). *Busseola fusca* moths have a preference for thick-stemmed plants for oviposition (Van Rensburg and Van den Berg, 1990), and high plant density promotes oviposition (Van Rensburg and Van den Berg, 1990).

The behavioural steps that lead to oviposition by a female moth generally follow a sequential pattern consisting of searching, orientation, encounter, landing, surface evaluation, and acceptance (Renwick and Chew, 1994). Visual or chemical cues, and most probably a combination of both, may trigger the general orientation of a *B. fusca* moth toward the host plant (Renwick and Chew, 1994; Calatayud *et al*., 2008). The insect can visually locate an upright plant stem by flying directly towards it and then landing. Shape is another visual cue that may play an important role in the general orientation towards the plant in nocturnally active lepidopterans, such as *B. fusca* (Renwick and Chew, 1994). The physiological status of the insect, such as its age and duration of deprivation of a suitable ovipositing site, also plays a role in host plant selection (Barton-Browne, 1993).

Stem borer larvae produce oral secretions such as regurgitated feed and those of salivary glands during feeding. These secretions contain herbivore-associated molecular patterns (HAMPs), which affect plant responses to herbivores as well pathogenic fungi or bacteria through the SA/JA cross-talk (Alborn, 1997; Musser *et al*., 2002; Mithöfer and Boland, 2008; Schäfer *et al*., 2011; Louis *et al*., 2013). Herbivore-associated contact due to feeding, oviposition and crawling also induce plant defences (Mithöfer *et al*., 2005; Hilker and Meiners, 2006; Peiffer *et al*., 2009; Bricchi *et al*., 2010). Another important stem borer behavior, defecation, also plays a role in plant-herbivore-pathogen interactions (Ray *et al*., 2015). Lepidopteran pests, such as the fall armyworm *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae), deposit substantial amounts of frass (excreta) within the enclosed whorl tissue surrounding their feeding site, where it remains for long periods of time (Ray *et al*., 2015). The frass is composed of molecules derived from the host plant, the insect itself, and associated microbes; and it provides abundant cues that alter plant defence responses (Ray *et al*., 2015). It has been observed that proteins from *S. frugiperda* frass induced wound-responsive defence genes in maize (Ray *et al*., 2015). Elicitation of pathogen defences by frass proteins was found to increase herbivore damage and reduce southern maize leaf blight caused by *Cochliobolus heterostrophus* (Drechsler) (Shanmugam *et al*., 2010; Ray *et al*., 2015). Moreover, *S. frugiperda* frass reduced the accumulation of maize herbivore defence gene transcripts and JA levels, while elevating the abundance of pathogen defence gene transcript (Ray *et al*., 2015).

Damage caused to maize plants by stem borers

Busseola fusca larvae cause damage to all maize plant parts (Calatayud *et al*., 2014). First generation *B. fusca* larvae feed within the whorls until the third instar thus destroying the growing point of the plant (dead-heart) (Van Rensburg *et al*., 1988a). They thereafter enter the plant stem (Van Rensburg *et al*., 1987b) where tunnelling results in extensive damage to internal stem tissue (Van Rensburg *et al*., 1988a). *Busseola fusca* larvae also cause direct damage to maize ears, and damage to plants after tasselling has the most important influence on yield (Van Rensburg *et al*., 1988a). The number of larvae per plant is, however, a poor indicator of expected yield loss (Van Rensburg *et al*., 1988d). After ballooning-off and feeding on whorl tissue, third instars bore into the stem and older instars may also migrate in search of more suitable host plants (Kaufmann, 1983). Only one larva usually feed per stem due to the cannibalistic nature of *B. fusca* larvae (Robertson, 2000). There are usually six larval instar stages, and larvae mature in about 35 days and pupate inside the stem for about 2-3 weeks. They make small exit holes before pupating to enable the emergence of the adult moth (Bijlmakers, 1989; Harris and Nwanze, 1992; Robertson, 2000).

Factors modulating survival and growth of *B. fusca* larvae

Environmental conditions

Weather conditions such as high wind speed affect moth flight activity and ballooning-off movement of larvae, while heavy rainfall results in drowning of neonate larvae (Van Rensburg *et al*., 1988b; Zalucki *et al*., 2002). Low rainfall, in contrast, results in reduced seasonal abundance of *B. fusca* moths (Van Rensburg *et al*., 1987a; Ebenebe *et al.*, 2000a), while the daily flight activity and survival of moths is enhanced by cool and humid conditions (Van Rensburg *et al*., 1987a). High levels of infestations can occur during years with adequate rainfall after older larval instars have penetrated the stem where they are protected from the adverse effects of rainfall (Van Rensburg *et al*., 1987a).

Production practices

Increased maize plant population enhances both the rates of dispersal and the survival of *B. fusca* larvae (Van Rensburg *et al*., 1988c; Van den Berg *et al*., 1991). A reduction in row width puts the adjacent plant rows within reach of more larvae through migration, resulting in increased plant damage (Van Rensburg *et al*., 1988c). The infestation potential of stem borers in maize is increased by the incidence of tillering and the availability of leaf sheaths that are suitable for oviposition (Van Rensburg and Van den Berg, 1990).

Management of maize stem borers

Biological control

Parasitoids that attack *B. fusca* larvae can be used as biological control agents. *Cotesia flavipes* Cameron (Hymenoptera: Braconidae) is the only exotic parasitoid that has established itself on maize in mainland Africa (Kfir *et al*., 2002). The short seasonal duration of maize farming provides only a 3-month habitat for stem borers and their natural predators. This lack of habitat stability results in the failure of parasitoids to establish and control stem borers (Hall and Ehler, 1979). Parasitoids could, in theory, have a better chance of establishing themselves in subsistence farming systems where maize is grown all year round, under irrigation or in small gardens. A parasitoid that has a wide host range of target species also has a better chance of establishment in a new area compared to a parasitoid with a narrow host range (Kfir *et al*., 2002). Climatic compatibility further influences parasitoid establishment. The temperate climate of South Africa has not been conducive to the establishment of parasitoids from tropical and sub-tropical regions (Skoroszewski and Van Hamburg, 1987; Kfir, 1994).

A positive relationship between any component of fitness of a species, and either numbers or density of conspecifics (Allee effect) (Fig. 4) (Berec *et al*., 2006), might explain why natural predators fail to establish as biological control agents (Hopper and Roush, 1993). Release of

a parasitoid which produces only male off-spring might decrease chances of mate finding, leading to possible extinction (Hopper and Roush, 1993). However, the braconids *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae) and *C. flavipes* can undergo inbreeding before dispersal to avoid the influence of Allee effects on their establishment on stem borers such as *B. fusca* (Arakaki and Gahana, 1986). In theory, a low-density equilibrium could be sustained in a deterministic equilibrium where the birth rate equals the death rate. However, given randomness, the population could be driven below the low density equilibrium and become extinct (Fig. 4) (Berec *et al*., 2006). An Allee effect can also be induced in insects to disrupt fertilisation by releasing sterile males (Lewis and Van den Driessche, 1993; Krafsur, 1998; Fagan *et al*., 2002). Models of sterile insect release showed that it introduces a density threshold into the population. The size of the threshold depends on the number or density of sterile insects released (Lewis and Van den Driessche, 1993).

Biological control has limitations since some insect species possess several defence strategies, such as an encapsulation mechanism by which oviposited parasitoid eggs are melanised by *B. fusca* larvae (Ngi-Song *et al.*, 1998; Mochiah *et al.*, 2002). A surface protein in the larvae possibly plays an important role in the encapsulation of parasitoid eggs (Mochiah *et al*., 2002). Stem borers also exhibit aggressive behaviour towards parasitoids through violent wriggling, biting and spitting (Potting *et al*., 1997). The endo-parasitic *C. flavipes* and the exo-parasitic *Goniozus indicus* Ashmead (Hymenoptera: Bethylidae) have a high probability of between 30-70% of being killed by defending stem borer larvae at each oviposition attempt (Potting *et al*., 1997). A biological control agent might attain higher success if conditions under which it is likely to induce an Allee effect are met, and understanding such conditions could be vital to the selection of relevant biological control agents (Taylor and Hastings, 2005).

Chemical control

Insecticide use on maize to control insects, including stem borers, is high in regions such as the European Union where national restrictions on *Bt* maize cultivation are enforced (Meissle *et al*., 2010). The most commonly used active ingredients for maize stem borer sprays in Europe include the pyrethroids and organophosphates, as well as oxadiazines, nicotinoids, carbamates and diflubenzuron (Meissle *et al*., 2010). The application of foliar insecticide spray formulations of Chlorpyrifos, Imidacloprid, Cypermethrin + Dimethoate, and Lambda-cyhalothrin were found to be effective in the control of *B. fusca* larvae in Nigeria. Moreover, the Imidacloprid-treated plants recorded the lowest degree of ear damage (Adamu *et al*., 2015), thereby indicating that Imidacloprid treatments could potentially reduce FER that is associated with stem borer damage. An insecticide mixture of Endosulfan and Deltamethrin applied to the whorl or the sides of plants was found to be effective in the control of *B. fusca*

in maize in South Africa (Van den Berg and Van Rensburg, 1996) due to the large proportion of *B. fusca* larvae present in the whorl and flag-leaf furl prior to tasselling (Van Rensburg *et al*., 1987b; 1988a).

Application of the pyrethroid insecticide, Beta-cyfluthrin, which acts as a granular stomach and contact insecticide, has been used to control *B. fusca* and *C. partellus* infestations in maize in Kenya (Beyene *et al*., 2011). Beta-cyfluthrin is effective in the whorl until shortly before tasselling, as stem borer larvae primarily feed in the whorls of plants until the 4th instar (Van Rensburg *et al*., 1987b). Treatment of other plant parts is often unnecessary (Van Rensburg *et al*., 1987b), and treatment after tasselling is ineffective since the active ingredients cannot penetrate the stem or ears where the stem borers reside (Van Rensburg *et al*., 1987b). Effective insecticide control can be achieved by targeting insecticide applications at young larval instars which is the most vulnerable stage of the *B. fusca* life cycle (Adamu *et al*., 2015). Control of *O. nubilalis* and the Mediterranean corn borer, *Sesamia nonagrioides* Lefebvre (Lepidoptera: Noctuidae), using Deltamethrin insecticide alone and in combination with the Tebuconazole fungicide treatment, did not result in a reduction in *Fusarium* mycoflora (Folcher *et al*., 2009). However, the insecticide and fungicide treatments were effective in significantly reducing fumonisin levels (Folcher *et al*., 2009).

Cultural practices

Crop residues provide a habitat for stem borer larvae to survive between seasons. To reduce stem borer densities, crop residues should be managed by removal and ploughing-in (Kfir *et al*., 2002). Furthermore, adaptation of planting dates to avoid periods of abundance of *B. fusca* moths, and planting of maize hybrids with a short growing season, can result in escape of damage (Van Rensburg *et al*., 1988c; Ebenebe *et al*., 2000b). Potential infestation of maize can be predicted with a fair degree of confidence if the planting date and size of the corresponding moth flights are taken into consideration (Van Rensburg *et al*., 1985). Modern agricultural practices are exacerbating pest control problems, as the removal of non-host plants from crop areas result in 'bare-soil' cultivation which increases the probabilities of an insect finding a host plant (Finch and Collier, 2000).

Push-pull habitat manipulation

A habitat management strategy, based on a stimulo-deterrent diversion or 'push-pull' approach (Miller and Cowles 1990), has been developed for cereal stem borer management in East Africa (Cook *et al*., 2007). In the push-pull system, trap plants are planted as sentinel plants around maize fields where they emit volatile compounds that attract invading adult moths, thus limiting moths from landing on maize plants (Khan *et al*., 2007). The trap plants provide the 'pull' in the 'push-pull' system and also serve as a refuge for the larvae's natural

predators. The 'push' is provided by plants which emit repellent chemicals that turn away moths from maize in an intercropping system (Khan *et al*., 2007).

The molasses grass, *Melinis minutiflora* P. Beauv., is used as a 'push' plant due to its emission of repellent volatile compounds that repel stem borer moths from maize. It also attracts *C. sesamiae* parasitoids, which results in increased larval parasitism of stem borers by *C. sesamiae* (Khan *et al*., 1997a). The leguminous species *Desmodium uncinatum* Jacq. (Fabaceae) also possesses repellent properties, and is planted between maize rows. It produces more volatiles than maize and is thus often selected as a repellent plant to facilitate orientation flight away from maize (Khan *et al*., 1997b; Chamberlain *et al*., 2006). In addition, *D. uncinatum* provides soil nutrient stability through nitrogen fixation and is a highly nutritious fodder crop (Khan *et al*., 1997b; 2007; 2008).

The characteristic chemical composition of the trap plant should be close to that of maize in order to attract (pull) as many *B. fusca* moths as possible (Calatayud *et al*., 2007). Both domestic and wild grasses can be planted to protect crops by attracting and trapping stem borers. Suitable trap crops include Napier grass and Sudan grass (*Sorghum x drummondii* [Nees ex. Steud.] Millsp. & Chase) (Khan *et al*., 1997b). Napier grass produces more volatiles than maize (Chamberlain *et al*., 2006) and is preferred for oviposition (Khan *et al*., 2007). Moreover, *B. fusca* larval survival is considerably lower on Napier grass than on maize due to the secretion of a sticky gum in Napier grass upon larval penetration. The gum traps the stem borers inside the stem, killing them or exposing them to natural predators (Khan *et al*., 2007; 2008). In addition, a much longer larval developmental period occurs on Napier grass, possibly due to the plant's low nutritional value or antibiosis effects on the larvae (Khan *et al*., 2006).

Plant resistance

Moderate resistance to *B. fusca* has been identified in the I137TN locally inbred line (Van Rensburg and Gevers, 1993) while the CML139 inbred line from the International Maize and Wheat Improvement Center (CIMMYT) was identified as the most resistant inbred line to *B. fusca* damage in South Africa (Van Rensburg and Van den Berg, 1995). Resistant inbred lines Mp704 and Mp706, originating from Mississippi, have been used as donor parents in recurrent selection programmes to develop locally-adapted germplasm with improved resistance to *B. fusca* in South Africa (Van Rensburg and Klopper, 2004). The Mississippi lines were tested locally and found to be highly resistant to *B. fusca* (Van Rensburg and Malan, 1990). However, the antibiosis present in the Mississippi lines was additively inherited and 35% heritable (Van Rensburg and Gevers, 1993). These lines, however, were found to be poorly adapted to South African conditions in hybrid combinations (Van Rensburg and Flett, 2010). The introgression of resistance from the Mississippi inbred lines into locally adapted germplasm using recurrent selection culminated in the release of 42 resistant inbred lines in South Africa during 2004

(Van Rensburg and Klopper, 2004). The release of these inbred lines was, however, eclipsed by the rapid uptake of *Bt* biotechnology (Van Rensburg and Flett, 2010).

Mwimali *et al*. (2015) indicated that recurrent selection was effective in accumulating favourable alleles for *B. fusca* and *C. partellus* stem borer resistance in two maize populations, CML395/MBRC5Bc and CML444/MBR/MDRC3Bc, in Kenya. Moreover, reduction in the injuries due to leaf feeding damage, cumulative stem tunnelling and number of exit holes contributed towards 43 and 70% net genetic gain in kernel yield under *B. fusca* and *C. partellus* infestation for the CML395/MBRC5Bc and CML444/MBR/MDRC3Bc populations, respectively (Mwimali *et al*., 2015).

The survival and relative growth rate of *B. fusca* larvae were found to be considerably higher on maize and wild sorghum (*S. arundinaceum*) than in five wild grasses (*P. purpureum, A. donax*, *S. megaphylla*, *P. maximum* and *Panicum deustum* Thunb.) due to high levels of silicon in wild grasses (Juma *et al*., 2015). The incorporation of silicon into an artificial *B. fusca* diet and in potted plants resulted in a significant reduction in *B. fusca* larval growth rate (Juma *et al*., 2015). Silicon disturbs lepidopteran larval performance by increasing leaf abrasion resulting in an increase in wear on insect mandibles that may physically deter larval feeding (Keeping *et al*., 2009; Kvedaras *et al*., 2009).

THE *FUSARIUM VERTICILLIOIDES* x *BUSSEOLA FUSCA* x MAIZE INTERACTION

Relationship of maize with *F. verticillioides* and *B. fusca*

Infection of maize plants with *F. verticillioides* influences their association with the sugarcane stem borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae), and *S. calamistis* stem borers (Cardwell *et al*., 2000). *Eldana saccharina, S. calamistis* and the maize ear borer *Mussidia nigrivenella* Ragonot (Lepidoptera: Pyralidae) were found to be attracted by and survived longer or had lower mortality on plants inoculated with *F. verticillioides* during the early vegetative growth stages (Schulthess *et al*., 2002). Early infection of maize by *F. verticillioides* can cause plant malformation associated with stem etiolation and multiple-ear phyllody that predisposes maize plants to infestation by lepidopteran and coleopteran pests (Cardwell *et al*., 2000). Moreover, the numbers of stem borers in the ear were significantly higher in *F. verticillioides*-inoculated plants, while FER symptoms were significantly correlated with the incidence of *F. verticillioides* infection (Cardwell *et al*., 2000).

Busseola fusca infestation resulted in an increased incidence of FER in maize (Flett and Van Rensburg, 1992). *Busseola fusca* creates wounds that can enable infection of maize plants with *F. verticillioides*. These wounds may lead to the transition from symptomless endophytism to necrotrophic pathogenicity (Rutherford *et al*., 2002). Infestation of maize with *E. saccharina* significantly increased the incidence and severity of stem rots (Bosque-Pérez

and Mareck, 1991), while infestation of maize with *O. nubilalis* has been shown to increase fumonisin production (Munkvold *et al*., 1997; 1999). Vectoring of ear rot fungi such as *F. verticillioides* by *B. fusca* has not been demonstrated. The larvae of other lepidopteran insects, such as *O. nubilalis*, however, was shown to serve as vectors in spreading *F. verticillioides* spores from maize leaf surfaces to kernels (Sobek and Munkvold, 1999).

Resistance in maize to *F. verticillioides* and *B. fusca*

Plant immunity to *F. verticillioides* (Starr *et al*., 2006) and *B. fusca* (Mwimali *et al*., 2015; 2016) has not been identified in maize hybrids. However, resistance to the pathogen (Mesterházy *et al*., 2012) and pest (Mwimali *et al*., 2015; 2016) can be achieved by resistance breeding. Polygenic sources of resistance to FER are known to occur in maize (Pérez-Brito *et al*., 2001), but are difficult to incorporate into hybrids. Plant defence mechanisms against *F. verticillioides* and stem borer damage involves morphological and biochemical responses, as determined by the genetic make-up of plants (Nabeshima *et al*., 2001).

Resistance mechanisms

Plant morphology: The morphology of maize hybrids may influence their susceptibility to *F. verticillioides* and *B. fusca*. Koehler (1942) found more than twice as much FER in ears with open husks when compared to closed husks. Growth and sporulation of *F. verticillioides* was also found only on exposed pollinated silks while significant growth reduction occurred on silks with husk cover (Duncan and Howard, 2010). Delayed silk senescence imposes a physical barrier between kernels and inoculum sources, and could contribute to the reduction in *F. verticillioides* infection (Santiago *et al*., 2015). The pericarp may also play an important role as a barrier against fungal infection (Santiago *et al*., 2015). Duncan and Howard (2010) reported that a resistant maize inbred line had closed stylar canals, which made it less prone to *F. verticillioides* infection than susceptible hybrids/inbred lines. Barros-Rios *et al*. (2011) demonstrated that the pith of resistant maize inbred lines had significantly higher concentrations of cell wall xylose than susceptible inbred lines.

Biochemical response: Three phytohormones; JA, SA and ET; are involved in plant defence responses to biotic and abiotic stresses (Antico *et al*., 2012). The SA and JA pathways are induced after plant infection with *Fusarium* spp., or after wounding by herbivorous insects such as *B. fusca*. SA is important in establishing resistance early on, while JA is important in facilitating resistance during later time points (Ding *et al*., 2011). Plants use ET to fine tune defences by prioritising JA induction over SA in response to multiple predators (Leon-Reyes *et al*., 2010). Despite the activation of the JA, SA and ET pathways to provide plant defences, herbivores are capable of hijacking plant defence signalling (Chung *et al*., 2013). Larvae of

the Colorado potato beetle *Leptinotarsa decemlineata* Say, for instance, release bacteria in their oral secretions that suppress anti-herbivore defences in tomato (*Solanum lycopersicum* L.) plants (Chung *et al*., 2013), and corn earworm, *Helicoverpa zea* Boddie, larvae secrete glucose oxidase in their saliva that reduces JA-regulated nicotine production in tobacco (*Nicotiana tabacum* L.) (Musser *et al*., 2002). The FB_1 produced by *F. verticillioides* is cytotoxic and induces programmed cell death in plants (Desjardins *et al*., 1995). It also suppresses JA levels in *Arabidopsis thaliana* (L.) Heynh, thereby increasing its vulnerability to herbivore attack (Zhang *et al*., 2015).

Phytoalexins are small toxic phenolic compounds that exhibit antifungal activity against phytopathogenic fungi. They are produced in plants following the induction of the JA, SA and ET signalling networks by plant pathogens (Turner *et al*., 2002). Two of these compounds, zealexins and kauralexins, exhibit both antifungal (Huffaker *et al*., 2011; Mao *et al*., 2016) and insect anti-feed activity in maize (Vaughan *et al*., 2015). The accumulation of phytoalexins was found to decrease in maize grown at elevated CO_2 levels following *F. verticillioides* stem inoculation (Vaughan *et al*., 2014). Elevated CO_2 levels also compromised the maize pathogenesis-related lipoxygenase (*LOX*)-dependent signalling, which influence the interactions between maize and *F. verticillioides* (Vaughan *et al*., 2014; 2016). Zealexins are ubiquitous in maize and are induced by biotic stimuli, including *O. nubilalis* herbivory (Huffaker *et al*., 2011). Other defensive plant metabolites, such as cyanogenic glycosides, are toxic to insects and pathogens following tissue damage and pathogen attack, respectively (Osbourn, 1996). Terpenoids and benzoxazinone glucosides further possess anti-feed or repellent properties that may affect the fecundity and oviposition of insect herbivores (Niemeyer, 1988; Sicker *et al*., 2000; Awmack and Leather, 2002). Terpenoids also exhibit potent antifungal activity through the disruption of ion homeostasis (Rao *et al*., 2010), and are produced constitutively as well as through induction by herbivore and pathogen attack (Keeling and Bohlmann, 2006; Frey *et al*., 2009). Benzoxazinone glucosides have been found to confer antifungal activity against *F. verticillioides in vitro* (Friebe, 2001). They are produced constitutively in plants, and can also be induced by fungal infection in maize (Oikawa *et al*., 2004; Huffaker *et al*., 2011).

Stem tunnelling by stem borers is negatively correlated with high concentrations of diferulates, 8-5-diferulate and *p*-coumarate esters (Barros-Rios *et al*., 2011). These esters generate ferulate-polysaccharide-lignin complexes that cross-link the cell wall (Buanafina, 2009). The deposition of diferulates in kernel, leaf, pith, rind and nodes was previously associated with maize resistance to *O. nubilalis* (Bergvinson *et al*., 1997) and *S. zeamais* (García-Lara *et al*., 2004).

A naturally occurring cysteine protease (Mir1-CP) from Antiguan maize germplasm has been shown to confer resistance to lepidopteran stem borers in maize (Mohan *et al*., 2008).

Mir1-CP is an enzyme that inhibits larval growth by attacking and permeabilising inner insect membranes. When larvae fed on Antiguan maize germplasm, the defensive Mir1-CP rapidly accumulated at the site of wounding (Mohan *et al*., 2008). The Mir1-CP protein could potentially be applied either singularly or in combination with *Bt* toxins (Mohan *et al*., 2008) as an insecticide or by means of plant breeding. The permeabilising of inner insect membranes can provide ready access to the *Bt* protein binding sites on the insect midgut microvilli, thereby increasing the *Bt* toxin activity.

Plant defence mechanisms against stem borer attack also include antixenosis and antibiosis (Butrón *et al*., 1998; 1999). Antixenosis reduces the probability of contact between potential herbivores and plants, while antibiosis reduces the growth and/or development of the larvae following the initiation of contact (Butrón *et al*., 1998). *Arabidopsis thaliana* plants are known to produce trichomes constitutively as well as through induction following artificial damage (Traw and Bergelson, 2003). Moreover, recent studies have shown that *S. frugiperda* frass releases biochemical cues in the form of proteins that increase herbivore performance while suppressing pathogen defences in maize (Ray *et al*., 2015). Despite maize plants initially detecting frass as a cue of herbivory, there is a reduction in anti-herbivore defences resulting in improved larval performance (Ray *et al*., 2015).

Molecular response: Gene expression plays a role in the modulation of plant resistance to biotic and abiotic stresses. Guo *et al*. (2015) indicated that the deletion of an *FvSO* gene; which is required for vegetative hyphal fusion, asexual growth, fumonisin production and virulence in *F. verticillioides*; resulted in the reduction of fungal growth, fungal biomass and fumonisin production. Plant acclimatisation and tolerance to biotic and abiotic stresses can also be achieved when glutathione and hydrogen peroxide act individually or in tandem in intracellular and systemic signaling systems (Foyer *et al*., 1997). However, gene expression associated with acclimatisation responses is sensitive to the reduction-oxidation potential of plant cells (Neill *et al*., 2002).

Studies in Italy have shown that ears of maize plants resistant to *F. verticillioides* have high levels of gene expression for the *LOX*-derived oxylipin genes that protect plants against oxidative stress (Maschietto *et al*., 2015). The attenuation of the maize lipoxygenase (*13-LOX*s) gene expression and JA production has been shown to correlate with reduced terpenoid phytoalexins and increased susceptibility to *F. verticillioides* infection (Vaughan *et al*., 2014). Moreover, Christensen *et al*. (2014) demonstrated that the *9-LOX* gene plays a key role in maize defence against *F. verticillioides* in diverse maize tissues.

Plant improvement

Resistance to FER involves hereditary traits that are determined by a large number of genes (Eller *et al*., 2008b). Moreover, resistance to *F. verticillioides* in one locality may not translate to resistance in other localities. This is because of differences in environmental conditions in different locations, which result in variations in FER and fumonisin production (Venturini *et al*., 2015). Individual *F. verticillioides* isolates have been found to be highly diverse in FER and fumonisin production (Danielsen *et al*., 1998; Reynoso *et al*., 2009; Schoeman, 2014; Qiu *et al*., 2015). Upon evaluation at different localities, over different seasons and on different maize hybrids in South Africa, the most virulent and toxigenic strains caused most disease and produced most fumonisins despite a significant location x isolate interaction (Schoeman, 2014). Of the 112 maize inbred lines tested for resistance to *B. fusca* and *C. partellus* in three localities in Kenya, 21 lines showed resistance to both *B. fusca* and *C. partellus* in at least two localities while only four lines showed resistance to both species across the three localities. Therefore, resistance to *B. fusca* and *C. partellus* is also affected by environmental conditions in different localities. Conventional breeding and biotechnology are thus utilised to develop insect- and pathogen-resistant hybrids.

Conventional breeding: Quantitative trait loci (QTLs) and marker-assisted selection (MAS) are being used to improve the efficiency of plant breeding (Perez-Brito *et al*., 2001; Robertson-Hoyt *et al*., 2006; Kozhukhova *et al*., 2007; Ding *et al*., 2008; Vermeulen, 2015). This is achieved by allowing for the transfer of specific genomic regions of interest and accelerating the recovery of the elite parent background (Eller *et al*., 2008a; Stevens, 2008). QTLs have been identified for resistance to FER (Perez-Brito *et al*., 2001; Robertson-Hoyt *et al*., 2006; Kozhukhova *et al*., 2007; Ding *et al*., 2008; Vermeulen, 2015). The QTL for resistance to the southwestern corn borer *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae) (Bohn *et al*., 1997; Groh *et al*., 1998) has been correlated with resistance to the sugarcane borer *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae) and *O. nubilalis* stem borers (Thome *et al*., 1992). Genome-wide selection has also been used to improve the efficiency and accuracy of selecting germplasm for specific quantitative traits as an alternative method to MAS (Zila *et al*., 2013) in breeding for FER and stem borer resistance (Samayoa *et al*., 2015).

Biotechnology: Planting *Bt* maize hybrids is an important mechanism for the management of stem borers of maize (Hellmich *et al*., 2008). *Bt* genes encode for the δ-endotoxin crystal proteins that are toxic to insects, killing them upon feeding. *Bt* maize hybrids have been found to be less prone to FER and fumonisin contamination than non-*Bt* maize hybrids after infestation with *O. nubilalis* stem borers in the USA (Munkvold *et al*., 1999). However, the interactive effect of *B. fusca* and *F. verticillioides* on FER and fumonisin production in *Bt* and

non-*Bt* hybrids has not been studied under South African farming conditions where *B. fusca* is the stem borer of economic importance.

Fumonisin-degrading genes from the black yeast strains *Exophiala spinifera* Carmichael and *Rhinocladiella atrovirens* Nannfeldt (Blackwell *et al*., 1999), as well from the *Sphingopyxis* sp. bacterial strain (Heinl *et al*., 2010), have been identified and sequenced. The possible application of these fumonisin-degrading genes for the generation of transgenic plants and fumonisin detoxification *in planta* has been suggested (Duvick, 2001). No studies on the application of these genes in transgenic plants have been reported (Hartinger and Moll, 2011).

The cultivation of *Bt* maize is on the increase in South Africa, as most producers prefer *Bt* maize hybrids for their convenience, yield protection and reduced need for chemical insecticides. Statistics have indicated that over 70% of maize planted in South Africa were genetically modified maize consisting of *Bt* insect resistance, herbicide tolerance and the combined (stacked) traits (James, 2012). The *MON810* gene is widely deployed for the control of stem borers in South Africa (Van Rensburg, 2007; Kruger *et al*., 2011) and has reduced stem borer damage in the USA (Munkvold *et al*., 1999; Dowd, 2000). However, *B. fusca* larvae have now developed resistance to first generation *Bt* hybrids (*MON810* gene) in South Africa (Kruger *et al*., 2011; 2012). This resistance to *Bt* maize is attributed to the compromised efficacy of the refuge strategy. Improper planting of the requisite non-*Bt* maize (refuge) results in a decrease in the population of *Bt*-susceptible individuals that are required to mate with moths from larvae that survive inside *Bt* maize fields in order to delay the onset of resistance (Kruger *et al*., 2012). Lepidopteran pests targeted with *Bt* maize include *O. nubilalis*, *B. fusca*, *H. armigera*, *H. zea*, *D. grandiosella*, common stem borer (*Papiapema nebris* Guenee) and armyworm (*Pseudaletia unipunctata* Haworth) (Van Wyk *et al*., 2008).

CONCLUSIONS

Maize production globally is affected by *F. verticillioides*, a fungus that causes FER and produces fumonisin mycotoxins. Factors contributing to FER and fumonisin production include drought, high temperatures, low pH, insect wounds, plant genotype and morphology, certain cultural practices, and poor post-harvest handling and storage. Maize production continues to be hindered by FER and fumonisins despite the use of biological methods, chemical methods, resistance mechanisms, conventional plant improvement, biotechnology, cultural practices and post-harvest handling storage management. In South Africa, maize is also affected by *B. fusca*, which causes damage by feeding on maize leaves, ears and stems. *Busseola fusca* is best controlled by planting of *Bt* maize, but larvae can also be controlled through the use of parasitoids and insecticides, altered cultural practices and push-pull habitat manipulation. Despite the use of control measures, *F. verticillioides* infection and fumonisin production

remains prevalent due to the inability to target the pest and pathogen with biocontrol agents and pesticides, and to obtain resistant maize hybrids (Munkvold and Desjardins, 1997).

Busseola fusca infestation and damage to maize ears has previously been associated with a higher incidence of FER (Flett and Van Rensburg, 1992). It is, however, not known if it is also associated with increased fumonisin production in maize kernels. In the USA, Europe and Argentina, higher fumonisin concentrations in non-*Bt* hybrids compared to *Bt* hybrids have been associated with *O. nubilalis* infestations (Munkvold *et al*., 1997; 1999; Bakan *et al*., 2002; Magg *et al*., 2002; Hammond *et al*., 2004; Barros *et al*., 2009). The introduction of the *MON810* gene into maize to control *O. nubilalis* (CERA, 2015) indirectly reduced FER and fumonisin production by *F. verticillioides*. Understanding the impact of *Bt* maize on FER and fumonisin production in South Africa, the only country that grows *Bt* maize on a commercial scale in Africa, will provide valuable information on the effect of *Bt* technology in food safety, with particular emphasis on FER and fumonisin production.

The effect *B. fusca* and mechanical wounding of maize ears on FER and fumonisin production will be determined in **Chapter 2**. Wounding of maize ears during and after silking by insects (Van Rensburg *et al*., 1988a) and hail damage (Le Roux and Olivier, 1996) are common events in South Africa. These wounds can provide an easy pathway for natural infection of plants with *F. verticillioides* (Odvody *et al*., 1997). Moreover, biotic wounding also induces plant stress which could promote FER and fumonisin production (Warfield and Gilchrist, 1999). A non-*Bt* hybrid will therefore be planted in two separate field trials to establish whether *B. fusca* damage and mechanical wounding have an effect on FER and fumonisin production.

Reduction of FER and fumonisin production in maize using *Bt* hybrids and conventional insecticide application to reduce *B. fusca* infestations will be investigated in **Chapter 3**. In this study, a *Bt* hybrid (*MON810* gene) and its insect-susceptible isohybrid will be inoculated with *F. verticillioides* and then infested with *B. fusca*. Fumonisin concentrations have been reduced in *Bt* hybrids that are toxic to *O. nubilalis* in the USA (Munkvold *et al.*, 1999), and it is thus important to also determine the effect of *Bt* hybrids infested with the African stem borer. The role of insecticides in reducing FER and fumonisin production in a conventional non-*Bt* hybrid will also be studied**.** Benfuracarb, a systemic insecticide, will be applied to maize seed prior to planting, while Beta-cyfluthrin, a non-systemic insecticide, will be applied weekly until tasselling. Beta-cyfluthrin and Benfuracarb are both widely used to control *B. fusca* larvae in South Africa (Ncube, 2008; J.B.J. van Rensburg, personal communication).

In **Chapter 4** the mycoflora associated with *B. fusca* frass will be determined, and the amount of fumonisin-producing *Fusarium* spp. present in *B. fusca* frass investigated. *Busseola fusca* larvae feed inside maize stems in close proximity with their own excreta, namely frass. Fungal species associated with stem borer frass might either act as non-pathogenic symbionts

or attract/repel insects that could either damage or protect the plant. The potential dissemination of *F. verticillioides* with *B. fusca* will be investigated by inoculating maize whorls with *F. verticillioides* followed by *B. fusca* larval infestation in greenhouse and field trials. The stems will be split open and the frass collected from feeding channels. Target DNA of fumonisin-producing *Fusarium* spp. in the frass will then be quantified. The dissemination of *F. verticillioides* spores by *B. fusca* larvae could result in the spread of FER, ear and stem rots.

The *F. verticillioides* x *B. fusca* interaction is an important dynamic in the maize production system in South Africa. Yet, there is a paucity of information on the effect of this interaction on FER development and fumonisin production in maize. There is also no information available on the fungal mycoflora in *B. fusca* frass and the role of *B. fusca* frass in the occurrence of maize diseases. This study seeks to fill these gaps by determining the role of *B*. *fusca* in the occurrence of FER and fumonisin production by *F*. *verticillioides.*

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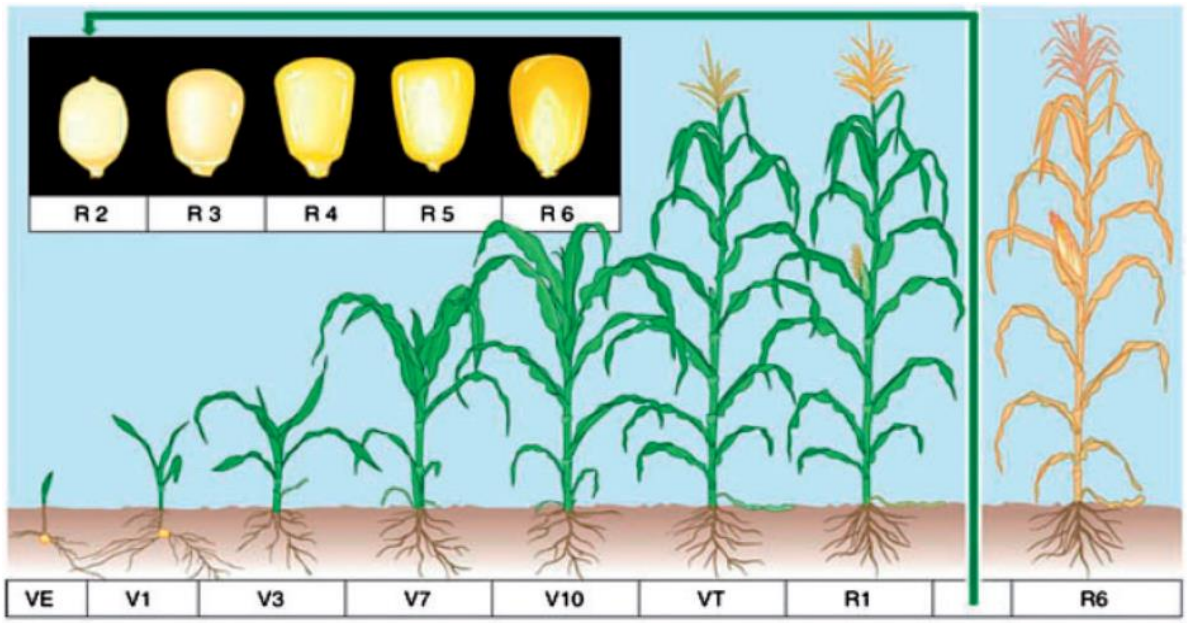

Figure 1. Vegetative (V) and reproductive (R) growth stages of maize. The vegetative stages begins with the VE stage at which the coleoptile is visible on the soil surface followed by the V1 stage at which the lowermost leaf has a visible leaf collar, and lastly, the VT stage, at which the last branch of the tassel is visible. The reproductive stages are: silking stage (R1); blister stage (R2) milky stage (R3); dough stage (R4); dent stage (R5); and physiological maturity stage (R6) (http://weedsoft.unl.edu/documents/GrowthStagesModule/Corn/Corn.htm).

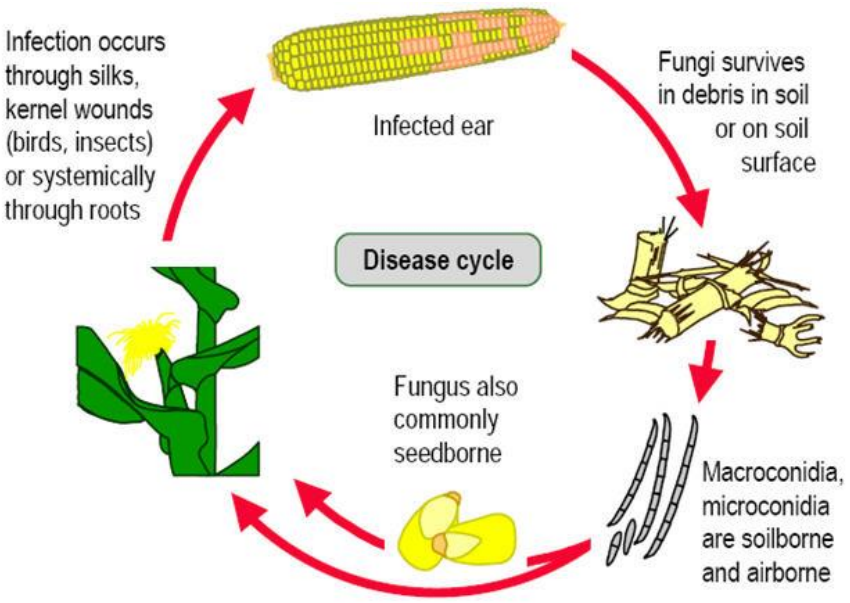

Figure 2. Life cycle of *Fusarium verticillioides* (https://www.pioneer.com)

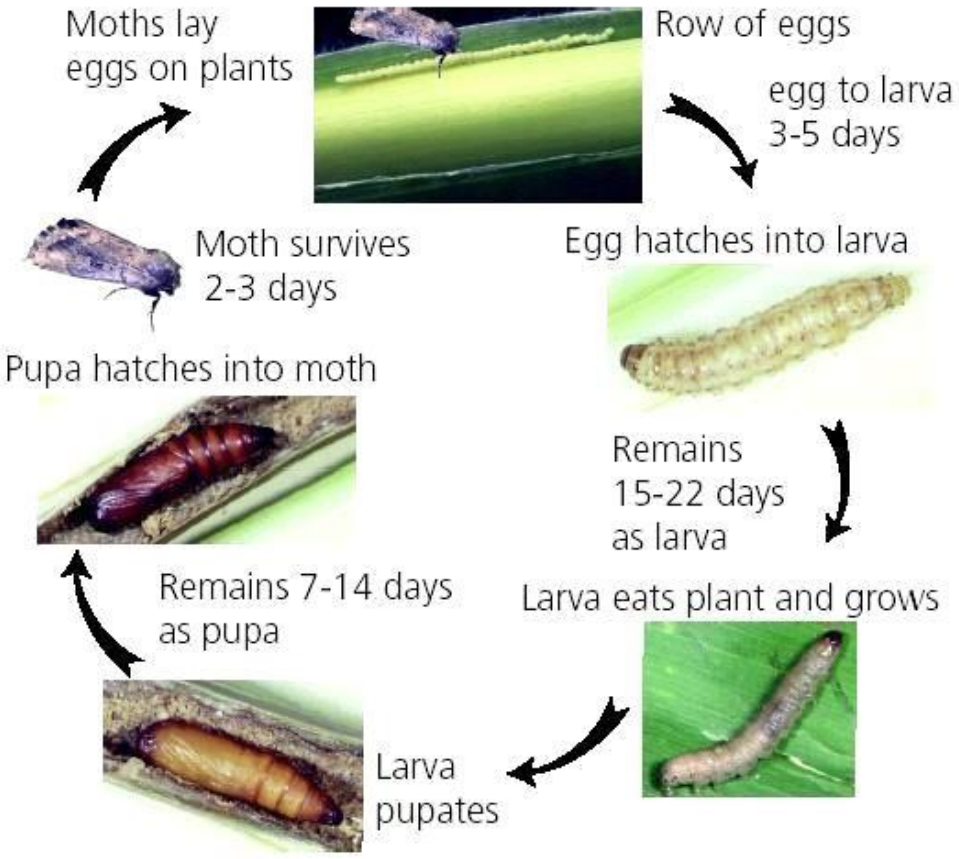

Figure 3. The life cycle of *Busseola fusca* (http://www.push-pull.net)

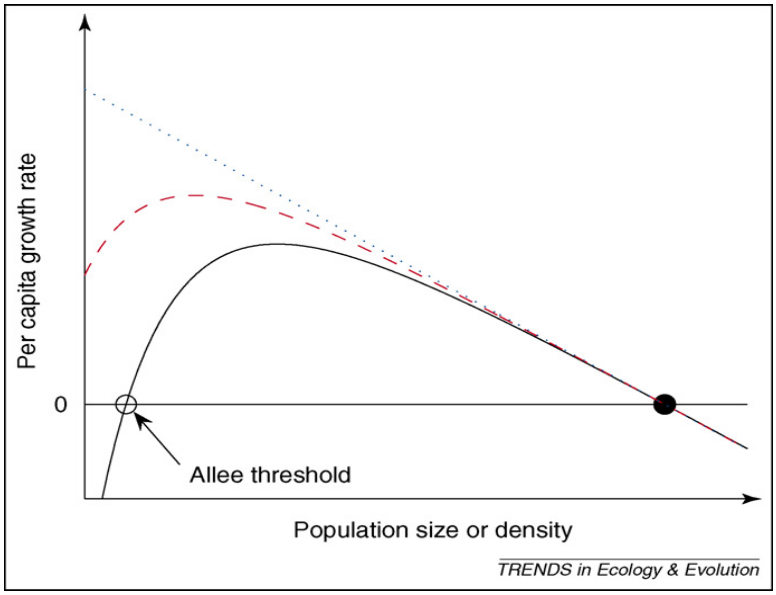

Figure 4. The relationships between the per capita population growth rate and either population size or density for negative density dependence (dotted curve), and weak (dashed curve) and strong (solid curve) Allee effects. In weak or strong Allee effects, the relationship is positive at low population sizes or densities, where positive density-dependent (Allee effect) mechanisms overpower negative density dependent (intra-specific competition) ones. It is negative at high population sizes or densities, where the converse is true (Berec *et al*., 2006). Allee threshold indicates the critical population size or density below which the per capita population growth rate becomes negative at which point the species becomes extinct (Berec *et al*., 2006).

CHAPTER 2

The effect of *Busseola fusca* and mechanical wounding on Fusarium ear rot development and fumonisin production in maize

ABSTRACT

Fusarium verticillioides and *Busseola fusca* are among the most significant biotic constraints to maize production in South Africa. In this study, the effect of *B. fusca* and mechanical wounding to maize ears on Fusarium ear rot (FER) development and fumonisin production was investigated. The effect of the interaction of FER and *B. fusca* damage was studied by inoculating maize ears with *F. verticillioides* isolate MRC826 and infesting plant whorls with aliquots of 10-15 neonate larvae at the 12th leaf stage prior to tasselling. For fungal inoculation, a 2-ml spore suspension containing 2×10^6 *F. verticillioides* spores ml^{-1} was injected into the silk channel of each primary ear at the blister stage. To simulate hail damage, maize ears were mechanically wounded at the blister stage with cork borers of different sizes and number of wounds, with and without *F. verticillioides* inoculation. Uninoculated, uninfested and undamaged control treatments were included. Field trials were conducted over three seasons using a randomised complete block design with six replicates per treatment. All primary ears were harvested at physiological maturity and percentage of kernels with FER, total fumonisin concentration (µg/g), larval damage in tunnel length and target DNA of fumonisin-producing *Fusarium* spp. (ng fungal DNA/0.5 mg milled sample) were quantified. In all seasons, most FER developed on ears inoculated with *F. verticillioides*. Regression analysis indicated that rainfall was negatively correlated with *B. fusca* damage, while heat units positively correlated with *B. fusca* damage. This study indicated that *B. fusca* infestation was not associated with higher FER incidence in two of the three seasons. Moreover, *B. fusca* infestation was not associated with high fumonisin production in any season. This indicates that *B. fusca* was not tightly correlated with FER or fumonisin production. This possibly is due to its characteristic feeding nature that causes sporadic wounds on maize ears, but also the strong impact of weather variability on FER. FER development and fumonisin production by *F. verticillioides* inoculum varied seasonally indicating the importance of environmental conditions on FER and fumonisin production. FER and fumonisin production significantly increased with the severity of cork borer wounding in both naturally infected and artificially inoculated maize ears. Therefore, the prevention of severe injuries to kernels; whether by mechanical damage or insects, should be considered as important in reducing FER and fumonisin production in maize.

INTRODUCTION

Maize (*Zea mays* L.) is the most important crop cultivated in South Africa for food consumption and as livestock feed (SAGIS, 2014; Visser, 2015; Kganyago, 2016). Production of maize in the country is, however, affected by a number of biotic and abiotic stresses. One of the most important biotic constraints is *Fusarium verticillioides* (Sacc.) Nirenberg (syn = *F. moniliforme* Sheldon), a fungus that causes Fusarium ear rot (FER), stem rot, root rot and seedling blight (Nelson *et al*., 1993; Leslie and Summerell, 2006). Infection of maize plants with *F. verticillioides* takes place from contaminated seed (Foley, 1962; Munkvold and Carlton, 1997), lateral root infection by soilborne spores (Oren *et al*., 2003), as well as through the silk channel by airborne spores (Munkvold and Carlton, 1997; Galperin *et al*., 2003). Wounding, caused by insects and mechanical damage, also provides an important entry point for the fungus (Flett and Van Rensburg, 1992; Munkvold *et al*., 1997; Robertson *et al*., 2011). FER symptoms become visible as single or groups of pinkish-red mouldy kernels, and/or pink or white streaks (White, 1999). *Fusarium verticillioides* can, however, sometimes infect kernels without showing any visible symptoms (Oren *et al*., 2003).

Infection of maize with *F. verticillioides* results in yield and quality losses of maize kernels (Kommedahl and Windels, 1981; Marasas *et al*., 1984). The most detrimental effect of the fungus is the production of fumonisin mycotoxins (Rheeder *et al*., 2002) that are harmful to human and animal health (Marasas, 1995). Fumonisins can cause human diseases such as neural tube defects (Missmer *et al*., 2006), and have been associated with oesophageal cancer in the Eastern Cape province of South Africa (Sydenham *et al*., 1990; Rheeder *et al*., 1992) and in the Cixian, Linxian and Shangqiu regions in China (Chu and Li, 1994; Yoshizawa *et al*., 1994). Various animal diseases, such as pulmonary edema in pigs (*Sus scrofa domestica* L.), can occur as a result of ingestion of fumonisin-contaminated feed (Haschek *et al*., 2001; Glenn, 2007). Toxicity to chickens (*Gallus gallus domesticus* L.) and broiler chicks also occurs as a result of consumption of fumonisin-contaminated feed. The most dramatic effect of maize feed contaminated with fumonisin is equine leukoencephalomalacia (ELEM), a neurotoxin disease of horses (*Equus feras caballus* L.) and donkeys (*Equus africanus asinus* L.) (Kellerman *et al*., 1990; Jovanović *et al*., 2015).

Environmental conditions prior to and during silking promote both FER and fumonisin production in maize (Warfield and Gilchrist, 1999; Janse van Rensburg, 2012). Adverse conditions such as drought stress and high temperature as well as cool, wet climate enhances the development of FER (Miller, 2001; Ngoko *et al*., 2001), while temperatures exceeding 26°C during the dough stage of kernel fill promote fumonisin production (Janse van Rensburg, 2012). Low soil moisture levels coupled with high soil and air temperatures during the later stages of ear maturity and dry down aggravate lateral splits in the kernel pericarp (Murillo-

Williams and Munkvold, 2008). This loss of kernel integrity exposes the kernel tissues to *F. verticillioides* infection and concomitant fumonisin production (Odvody *et al*., 1997). Hybrids grown outside their range of adaptation are more susceptible to *F. verticillioides* infection and concomitant fumonisin production (Shelby *et al*., 1994). Moreover, hybrid x season and season x location interactions are significant sources of variation for FER and fumonisin production in maize (Schoeman, 2014; Venturini *et al*., 2015).

Infection of maize with *F. verticillioides* has been reported to be increased due to damage caused by stem borers. Infestations with *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae), for instance, resulted in higher fumonisin concentrations in non-*Bt* hybrids in the USA (Munkvold *et al*., 1997; 1999; Hammond *et al*., 2004), Europe (Bakan *et al*., 2002; Magg *et al*., 2002) and Argentina (Barros *et al*., 2009). In South Africa, *Busseola fusca* Fuller (Lepidoptera: Noctuidae) was associated with a high incidence of FER (Flett and Van Rensburg, 1992), but there is no data available on its influence on fumonisin production. *Busseola fusca* is regarded as the most injurious pest of maize in South Africa (Van Rensburg *et al*., 1988a). Its larvae reduce maize yield through feeding on leaves, destruction of the growing point of the plant (dead heart), and by causing stem damage (Van Rensburg *et al*., 1988a). Female moths oviposit their eggs behind the vertical edges of leaf sheaths of pre-tasselling plants (Van Rensburg *et al*., 1987; Calatayud *et al*., 2014), but oviposition can also occur after tasselling when younger plants are not readily available (Van Rensburg *et al*., 1987). The eggs hatch into larvae that feed on maize plants before turning into pupae, after which adult moths will emerge to complete the life cycle (Calatayud *et al*., 2014).

Information on the effect of wounding on *F. verticillioides* infection of maize ears is lacking. Injuries in maize production systems can occur because of hail damage (Robertson *et al*., 2011), herbicide damage (Huang *et al*., 2012), mechanical cultivation equipment (Bricknell *et al*., 2008) and bird damage (Klosterman *et al*., 2012). In South Africa, the effect of hail damage to FER and fumonisin production is particularly important, as the main maize-producing areas of the country are often severely affected by hailstorms (SAIA, 2014). Direct damage to maize ears occur as a result of hail damage during the later reproductive stages of maize (Klein and Shapiro, 2011). The force of the hailstorm results in damage to maize kernels beneath the husks (Klein and Shapiro, 2011), thereby exposing kernels to natural infection with *F. verticillioides*. Hail damage to kernels increased the risk of FER and fumonisin production in the USA (Robertson *et al*., 2011), but this has not been investigated under South African farming conditions.

The aim of this study was to determine the effect of *B. fusca* and mechanical wounding to maize ears on FER and fumonisin production. Mechanical wounding of maize ears was performed to simulate hail damage, which frequently occurs in the maize-producing region in South Africa (Le Roux and Olivier, 1996).

MATERIALS AND METHODS

Experimental design

Two field trials with a conventional non-*Bt* commercial maize hybrid (PAN6723) were planted over three growing seasons in South Africa. One of the trials was used to determine the effect of biological damage caused by *B. fusca* on FER and fumonisin production, and the other to investigate the effect of mechanical wounds on FER and fumonisin production. The *B. fusca* damage field trial was planted during the 2008/09, 2010/11 and 2011/12 growing seasons, and the mechanical wounding trial during the 2009/10, 2010/11 and 2011/12 growing seasons.

Planting was done under dry land conditions at the ARC-Grain Crops Institute (ARC-GCI) experimental farm in Potchefstroom, North West province, South Africa (26˚73'60.7"S; 27˚07'55.3"E) with hybrid seeds purchased from Pannar Seeds (Greytown, South Africa). Soil samples were collected and analysed at the ARC-Institute of Industrial Crops Soil Analysis Laboratory in Rustenburg, South Africa to determine the quantity of fertiliser that needed to be applied in each season. Pre-emergence (S-metolachlor) and post-emergence (thiadiazine) herbicides were applied for weed control according to the manufacturer's instructions. The *B. fusca* trial was planted on 0.5 ha with 5-m rows, and the mechanical damage trial on 1 ha with 20-m rows. In both trials the intra-row spacing was 30 cm and inter-row spacing 1.5 m. The experimental rows had two border rows on each side to reduce inter-row interference.

The *B. fusca* trials consisted of four treatments with six replicates, planted in a randomised complete block design. The treatments were: *B. fusca* infestation only, *F. verticillioides* inoculation only, *F. verticillioides* inoculation and *B. fusca* infestation combined, and a control treatment with neither *B. fusca* nor *F. verticillioides*. For the mechanical wounding trial, different levels of wounding were inflicted to maize ears by using cork borers. The following treatments were used with and without *F. verticillioides* inoculation: wounding with cork borers with diameters of 1.59-, 1.75-, 2.23- and 2.39 cm. The 1.59-cm-diameter cork borer was used to stab through the husk to create a single wound. To increase the severity of wounding, five wounds were made with each of the 1.75-, 2.23- and 2.39-cm-diameter cork borers on maize ears at the blister stage. An unwounded control treatment was included.

Infestation with *B. fusca* and inoculation with *F. verticillioides*

Neonate *B. fusca* larvae were mass-reared at a facility at the ARC-GCI in Potchefstroom according to the method described by Van Rensburg and Van Rensburg (1993). Aliquots of 10-15 neonate larvae were deposited into the whorl of each plant (Flett and Van Rensburg, 1992) at the 12th leaf growth stage before tasselling by using a mechanical applicator (Van Rensburg and Van Rensburg, 1993). Inoculation with *B. fusca* larvae was done at this stage

to increase the likelihood of ear damage before the larvae migrate to the stems (J.B.J. van Rensburg, personal communication).

A high fumonisin-producing *F. verticillioides* isolate MRC826 (Gelderblom *et al*., 2001); obtained from the Medical Research Council (MRC) in Tygerberg, South Africa; was used to inoculate maize ears. The fungus was cultured on potato dextrose agar (PDA) for 4 days at 25°C. Two agar plugs, taken from an actively-growing culture, were then transferred into 100 ml sterile Armstrong *Fusarium* medium (Booth, 1971) in 200-ml Erlenmeyer flasks, and incubated on a rotary shaker at 100 revolutions per min (rpm) at 25°C. *Fusarium verticillioides* spores were harvested after 4 days, filtered through two layers of sterile cheesecloth, and centrifuged at 1 000 rpm using a swinging bucket rotor in a Hermle Z400® centrifuge (Hermle Labortechnik, Wehingen, Germany) for 10 min. The spores were washed in 100 ml sterile distilled water that was previously de-ionised using a PureLab Ultra® machine (Elga Process Water, Marlow, UK). The suspension was re-centrifuged for 10 min before decanting the supernatant. This step was repeated twice before 100 ml of de-ionised sterile water was added to the washed spores, and the concentration of the conidial spore suspension adjusted to 2×10^6 spores ml^{-1} using a Axioskop® Routine microscope (Carl Zeiss, Oberkochen, Germany) and a Fuchs Rosenthal haemocytometer (Hawksley, London, UK). Tween 20 surfactant (Fischer Biotech, Fairlawn, NJ, USA) was added to the spore suspension at a rate of 30 µl L^{-1} of spore suspension to minimise clumping of spores.

Maize ears were inoculated with *F. verticillioides* by injecting 2 ml of the *F. verticillioides* conidial spore suspension into the silk channel of each primary ear at the silking stage for the *B. fusca* damage trial, and at the blister stage for the mechanical wounding trial. A cattle injector, fitted with an 18 G × 1.5 inch (1.20 × 38 mm) Terumo needle (sterile, nontoxic, and nonpyrogenic) (Senwes, Potchefstroom, South Africa), was used for silk inoculations in both trials, as described by Small *et al*. (2012). At physiological maturity, all primary maize ears in each experimental row were hand harvested and evaluated for FER and *B. fusca* damage. Maize ears from each experimental row were mechanically threshed and a 1-kg sample was taken from which a subsample of 250 g was ground into a fine powder using a Cyclotec sample mill (Foss Tecator, Hoganas, Sweden) with a 1-mm mesh sieve. The milled samples were subsequently used to quantify fumonisin-producing *Fusarium* spp. and fumonisin concentration in the maize kernels.

Fusarium ear rot and *B. fusca* damage ratings

FER symptoms were visually rated on each ear and expressed as percentage ear infection (Ennerson and Hunter, 1980). All ears in each experimental row (replicate) were rated individually, and mean FER was calculated by adding the ratings per row and dividing the total by the number of ears for each experimental row. *Busseola fusca* damage was rated by

measuring the cumulative feeding tunnel length (cm) (Chery *et al*., 2004) on each ear. The mean *B. fusca* damage per ear was then calculated in the same manner as that of FER. *Busseola fusca* larvae formed both symptomatic *Fusarium*-infected and non-symptomatic tunnels on the ears. The symptomatic *Fusarium*-infected tunnels were included in FER ratings, while non-symptomatic tunnels were not included (Fig. 1).

Quantification of fumonisin-producing *Fusarium* spp.

A quantitative real-time PCR (qPCR) analysis for the measurement of target DNA of fumonisin-producing *Fusarium* spp. (fungal biomass) was performed on milled maize kernels. All kernels (both symptomatic and non-symptomatic) were milled for each replicate. This was done by using primers and probes designed for the polyketide synthase gene (*FUM1*); a conserved region responsible for fumonisin synthesis in *Fusarium* spp. (Waalwijk *et al*., 2008). Fungal DNA was extracted from a 0.5-mg sample of ground maize kernels with a DNeasy Plant Mini Kit® (Qiagen, Venlo, Netherlands). All DNA samples were then diluted to a concentration of 10 ng with molecular grade water (Melford Laboratories, Ipswich, UK). A NanoDrop 2000c spectrophotometer (NanoDrop, Wilmington, DE, USA) was used to quantify DNA concentration, as described by Janse van Rensburg *et al*. (2016). Extracted and diluted DNA was frozen at -20˚C until analysis.

qPCR analysis was performed using clear, low profile 96-well PCR plates (Bio-Rad Laboratories, Hercules, CA, USA). Assays were performed in a total volume of 25 µl consisting of 4 µl of sample DNA and 21 µl master mix [2.125 µl *FUM1*- probe (1 µM), 0.875 µl Taqfum-2F (0.33 µM), 0.875 µl Vpgen-3R (0.33 µM), 12.5 µl Sensimix® (Bioline, London, UK) and 4.625 µl molecular grade water (Melford Laboratories)]. The probe and primers used were as previously described by Waalwijk *et al*. (2008). Five DNA standards were prepared from *F. verticillioides* MRC826, which was used as a fungal reference culture, to construct a standard curve. The standards had a DNA concentration of 10 ng, 1 ng, 100 pg, 10 pg and 1 pg. qPCR reactions were performed in triplicate for each standard. The real-time PCR was performed on a CFX96™ Real-Time System (Bio-Rad Laboratories) using the following cycling conditions: 95˚C for 10 min, followed by 40 PCR cycles at 95˚C for 10 s, 60˚C for 30 s, and 72˚C for 10 s. An iCycler™ iQ Optical System Software Version 3.0a (Bio-Rad Laboratories) was used to quantify target DNA of fumonisin-producing *Fusarium* spp. The efficiency of the qPCR runs ranged from 96.1-99.9%, while the R^2 ranged from 0.992-0.997.

Quantification of fumonisins

Fumonisin analysis was performed at the ARC-GCI Mycotoxin Laboratory, Potchefstroom, using High-Performance Liquid Chromatography (HPLC) (Waters Corp., Milford, MA, USA). The FumoniTest™ HPLC procedure (Vicam®, Watertown, MA, USA) was performed on 50-g

sub-samples obtained from 250-g maize kernel samples. The FumoniTest™ HPLC procedure quantifies individual concentrations of fumonisin B_1, B_2 and B_3. Fumonisins were derivatised with *o*-phthaldialdehyde and assayed using an HPLC Symmetry® C18 column (Waters Corp.) with dimensions of 3.9 × 150 mm and a volume of 4 µl. The mobile phase consisted of methanol (Romil, Cambridge, UK) : 0.1 M NaH_2PO_4 (Merck, Johannesburg, South Africa) (77:23, v/v), adjusted to pH 3.3 with *o*-phosphoric acid (Merck). The flow rate was 0.8 ml/min. A scanning fluorescence detector (Waters® 474) with an excitation of 335 nm and emission of 440 nm was used. A multi λ fluorescence detector and Breeze™ software (Waters Corp.) were part of the HPLC system. The retention times were approximately 5.5, 11.5 and 12.5 min for FB_1, FB_2 and FB_3, respectively. The limit of detection was 0.016 µg/g, and five fumonisin standards (MRC) containing total fumonisin (B_1, B_2 and B_3) concentrations of 2, 5, 10, 15 and 20 µg/g, respectively, were included in each run.

Meteorological data

Rainfall, heat units, temperature and humidity data for the 2008/09-2011/12 seasons in Potchefstroom was obtained from the Agricultural Research Council-Institute of Soil, Climate and Water (ARC-ISCW), Pretoria, South Africa on a monthly basis between January and April. The period between January and April is important in the FER and fumonisin context because it covers the silking up to dent stage at which FER and fumonisin production occurs (Murillo-Williams and Munkvold, 2008; Janse van Rensburg, 2012). Heat unit systems quantify the thermal environment of crops and relate crop growth and development to local weather/climate conditions (Brown, 2013).

Statistical analyses

An analysis of variance (ANOVA) was performed on the data for FER, fumonisin production and quantity of target DNA of fumonisin-producing *Fusarium* spp. for both *B. fusca* damage and mechanical damage trials using GenStat 14th edition (VSN, International, Hemel Hempstead, UK) at Tukey's 95% confidence interval.

In the *B. fusca* damage trial, simple regression analyses between the cumulative tunnel length caused by *B. fusca* and FER, fumonisin production, quantity of target DNA of fumonisin-producing *Fusarium* spp., mean rainfall, mean heat units, and mean temperature were performed using Statgraphics® 5 Plus (Statpoint Technologies Incorporated, Warrenton, VA, USA) across three seasons. Simple regression analyses were also performed between fumonisin production and FER, quantity of target DNA of fumonisin-producing *Fusarium* spp., and mean rainfall using Statgraphics® 5 Plus (Statpoint Technologies) across three seasons. Simple regression analyses between FER and heat units as well as mean rainfall were further performed using Statgraphics® 5 Plus (Statpoint Technologies) across three seasons.

In the field trial to determine the effect of mechanical wounding on FER and fumonisin production, simple regression analyses between fumonisin production and FER, and quantity of target DNA of fumonisin-producing *Fusarium* spp.; as well as between FER and quantity of target DNA of fumonisin-producing *Fusarium* spp.; and between quantity of target DNA of fumonisin-producing *Fusarium* spp. and mean temperature were performed using Statgraphics® 5 Plus (Statpoint Technologies) across three seasons.

RESULTS

Busseola fusca and *F. verticillioides* effects on FER, stem borer damage, fumonisin production and quantity of target DNA of fumonisin-producing *Fusarium* spp.

Inoculation of maize ears with *F. verticillioides* resulted in significantly higher levels of FER compared to the control treatment. However, there was no significant differences in the levels of FER in the maize ears that were only inoculated with *F. verticillioides* and those where only *B. fusca* infestation was performed in the 2008/09 season. There were no significant differences across treatments in 2010/11 (P<0.05), but in the 2011/12 season, *B. fusca* infestation alone was associated with a higher incidence of FER compared to the control treatment and maize ears inoculated with only *F. verticillioides* (Table, 1). The joint *F. verticillioides* and *B. fusca* inoculation in the 2008/09 season resulted in significantly more FER compared to inoculation with *F. verticillioides* and infestation with *B. fusca* only, but not during the 2010/11 and 2011/12 seasons (Table 1). FER development in the *F. verticillioides*-inoculated plants was significantly more in the 2008/09 compared to the 2011/12 season, but there were no significant difference among the control treatments over the three seasons (Table 1).

The cumulative *B. fusca* tunnel length during the 2008/09 season was significantly longer in all of the treatments when compared to the non-treated control (Table 2). This included ears that were not infested with *B. fusca*, but only inoculated with *F. verticillioides*. *Busseola fusca* damage in 2008/09 was also significantly more in the *F. verticillioides*-inoculated and *B. fusca*-infested plants than in seasons 2010/11 and 2011/12 (P<0.05). In the 2010/11 and 2011/12 seasons, the damage caused by *B. fusca* to maize ears did not differ significantly between the infested and control plants (Table 2).

Fusarium verticillioides inoculation, with and without *B. fusca* infestation, significantly increased fumonisin production compared to the *B. fusca* alone and control treatments (P<0.05) in the 2008/09 and 2010/11 seasons, but not in the 2011/12 season (Table 3). Significantly more fumonisins were produced by *F. verticillioides* in the 2008/09 season compared to the 2010/11 and 2011/12 seasons, and in the *F. verticillioides* x *B. fusca* treatment in the 2008/09 season compared to the 2011/12 season. There were no significant

differences in fumonisin production in the control treatment across all seasons, and the treatments had no effect on fumonisin levels in the 2011/12 season (Table 3). Neither *B. fusca* nor *F. verticillioides*, or a combination thereof, affected the colonization of maize ears with fumonisin-producing *Fusarium* spp. significantly (P>0.05) (data not presented).

Mechanical ear wounding effect on FER, fumonisin production and quantity of target DNA of fumonisin-producing *Fusarium* spp.

A significant seasonal main effect was observed for FER development on maize ears that were mechanically wounded with cork borers. FER was significantly more severe in 2009/10 than in the other seasons, and also significantly more severe in 2010/11 than in the 2011/12 season (Table 4). The disease increased significantly when wounds were made with cork borers larger than the 1.59-cm cork borer, and when multiple wounds were made (Table 5).

Fumonisin production in maize ears that were mechanically wounded showed significant seasonal (Table 6) and treatment (Table 7) main effects. Fumonisin levels were significantly higher during the 2009/10 and 2010/11 seasons than the 2011/12 season (Table 6). All wounded *F. verticillioides*-inoculated and -uninoculated maize ears were significantly more contaminated with fumonisins compared to the controls, with one exception. Fumonisin production in the non-inoculated ears wounded with the 1.59-cm cork borer, which was the smallest diameter, did not differ significantly from the control (Table 7). The results further showed that fumonisin production increased when the severity of mechanical wounding increased.

The interaction between growing season and wounding method used significantly affected the quantity of target DNA of fumonisin-producing *Fusarium* spp. (fungal biomass) in maize kernels (Table 8). Fungal biomass in kernels harvested in the 2011/12 season, whether wounded and treated with *F. verticillioides*, did not differ significantly from the untreated controls. Fungal colonisation of maize ears wounded with the 1.59-cm cork borer, whether inoculated with *F. verticillioides* or not, also did not differ from non-wounded ears in the other two seasons studied (2009/10 and 2010/11). When ears were wounded with the 1.75-cm cork borer and inoculated with *F. verticillioides* during these seasons, fungal contamination was significantly more in the inoculated than the uninoculated ears in 2009/10, but not in 2010/11 (Table 8). Wounding treatments alone did not significantly increase the quantity of target DNA of fumonisin-producing *Fusarium* spp. during the 2009/10 and 2011/12 seasons. In the 2010/11 season, however, fungal DNA was significantly more when ears were wounded multiple times with 2.23- and 2.39-cm cork borers.

Meteorological data

The highest mean rainfall of 137.4 mm and cool conditions with heat units of -110.6 were recorded in the 2009/10 season, followed by the 2010/11 season with mean rainfall of 89.8 mm and cool conditions with heat units of -101.1 from January to April (Fig. 2). The 2011/12 season had mean rainfall of 74.2 mm and heat units equal to -58. The 2008/09 season had the lowest mean rainfall of 53.7 mm coupled with highest heat units of -35.7 (Fig. 2).

Simple regression analyses

The cumulative tunnel length caused by *B. fusca* correlated moderately with FER (r = 0.43), fumonisin production (r = 0.52) and quantity of target DNA of fumonisin-producing *Fusarium* spp. (r = 0.41). Moreover, *B. fusca* damage had a strong negative correlation with mean rainfall (r = -0.73) and a strong positive correlation with mean heat units (r = 0.62), while also weakly correlating with mean temperature (r = 0.39) following artificial infestation of maize with *B. fusca* larvae and inoculation with *F. verticillioides* as determined by simple regression analyses (Fig. 3).

Fumonisin production correlated moderately with FER (r = 0.56) and the quantity of target DNA of fumonisin-producing *Fusarium* spp. (r = 0.38). The correlation between fumonisin production and rainfall, however, was negative (r = -0.27) (Fig. 4). FER was not highly correlated with heat units and mean rainfall, with correlation coefficients of 0.24 and -0.28, respectively (Fig. 4). Mean relative humidity did not significantly correlate with any of the parameters tested (data not presented).

Fumonisin production correlated significantly with FER (r = 0.47) and the quantity of target DNA of fumonisin-producing *Fusarium* spp. (r = 0.48) in a field trial to determine the effect of mechanical wounding on FER and fumonisin production. There was also a moderate correlation between FER and the quantity of target DNA of fumonisin-producing *Fusarium* spp. (r = 0.48). The quantity of target DNA of fumonisin-producing *Fusarium* spp. correlated poorly with mean temperature, with a correlation coefficient of 0.19 (Fig. 5).

DISCUSSION

The development of FER and the production of fumonisins in maize in this study were significantly affected by the growing season in which field trials were conducted. This was particularly true for the 2011/12 season, in which FER and fumonisins produced were significantly less than in the previous three seasons. In 2011/12, FER and fumonisin production in *F. verticillioides*-inoculated plants treated with *B. fusca* also did not differ significantly from the untreated control plants. Moreover, the quantity of target DNA of

fumonisin-producing *Fusarium* spp. in *F. verticillioides*-inoculated plants wounded with cork borers also did not differ significantly from the untreated control plants in 2011/12. In all the other seasons, however, *F. verticillioides* inoculation and cork borer damage resulted in significant increases in FER and fumonisin production in maize. The reason for the lack of FER and fumonisin production during the 2011/12 growing season is unclear, but could potentially be contributed by environmental effects on the fungus as indicated by non-significant differences in the quantity of target DNA of fumonisin-producing *Fusarium* spp. in all treatments in the mechanical wounding trial. The highest levels of fumonisin occurring in the cool, wet seasons but not in the hot dry season of 2011/2012 indicate that cool and wet conditions are also favourable for fumonisin production (Ngoko *et al*., 2001; 2002).

This study showed that *B. fusca* infestation was not associated with a higher incidence of FER and fumonisin production, even in the 2008/09 season where there was significant *B. fusca* damage to maize ears. Moreover, moderate correlations between *B. fusca* damage and FER, fumonisin production and the quantity of target DNA of fumonisin-producing *Fusarium* spp. also indicate the low impact of *B. fusca* damage on fungal colonization, and, consequently on FER and fumonisin production. An increase in the severity of cork borer damage, however, resulted in significant increases in FER and fumonisin production, suggesting that large wounds contribute to increased FER and fumonisin. This further suggest that the control of *B. fusca* will not necessarily result in the control FER and fumonisin since FER and fumonisin production depend on infection by the fungus, and severe biotic as well as abiotic wounding will increase FER and fumonisins.

Tunnels caused by *B. fusca* feeding in the 2008/09 season were significantly more severe than in 2010/11. Alternatively, survival of *B. fusca* larvae in 2010/11 was less than in 2008/09. Higher rainfall is known to result in the drowning of young larval instars (Van Rensburg *et al*., 1988b). *Busseola fusca* growth and survival could also have been affected by the larval parasitoid, *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae), which parasitise *B. fusca* larvae in South Africa (Kruger *et al.*, 2008), or *Beauveria bassiana* (Balsamo) Vuillemin, which was previously shown to be a mortality factor of *B. fusca* (Van Rensburg *et al*., 1988c; Maniania *et al*., 2011). Moreover, *B. fusca* tunnel length was increased when *F. verticillioides* alone was inoculated in 2008/09. This indicates that inoculation with *F. verticillioides* might have stimulated natural *B. fusca* infestation, and this could have been affected by seasonal conditions since the 2008/09 season was the most dry and hot season. This is similar to findings by Schulthess *et al*. (2002) that indicated that *Eldana saccharina* Walker (Lepidoptera: Pyralidae), *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) and *Mussidia nigrivenella* Ragonot (Lepidoptera: Pyralidae) were found to be attracted by and survived longer or had lower mortality on plants inoculated with *F. verticillioides* during the early vegetative growth stages.

Multiple large wounds created by cork borers resulted in significantly more FER symptoms and fumonisin production, irrespective of artificial *F. verticillioides* inoculation of maize ears. This indicates that consistent wounding that opens up husk coverage and exposes maize kernels; caused by factors such as hail damage, damage by implements and bird damage; could be important entry points for *F. verticillioides* that might result in FER and fumonisin production in maize kernels. The combined inoculation of maize ears with *F. verticillioides* and infestation of whorls with *B. fusca* during the 2010/11 season did not result in a significant increase in FER compared to the non-inoculated control treatment. This could be due to non-symptomatic infection by *F. verticillioides* of maize kernels during this season. Moreover, FER development and fumonisin production by *F. verticillioides* varied seasonally indicating the importance of environmental conditions on FER and fumonisin production. This study further indicated that the severity of wounding is an important contributing factor modulating FER and fumonisin production.

Prevention of severe injuries to kernels, either by insects or by mechanical wounding, is key in mitigating FER and fumonisin production in maize. Stem borer infestation alone would not influence FER and fumonisin. Prevention of large wounds, however, would reduce FER and fumonisin, as these provide entry points for the fungus (as was demonstrated in the lack of difference in FER and fumonisin production between *F. verticillioides*-inoculated and non-inoculated mechanical wounds). Therefore, hail, birds, and even substantial larval feeding produce large wounds where *F. verticillioides* can infect, thereby increasing FER and fumonisin production. While it is impossible to prevent hail, it might be possible to reduce large insect wounds, bird wounds and mechanical wounding in order to minimise FER and fumonisin production in maize ears.

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Table 1. Fusarium ear rot development (%) in a non-*Bt* maize hybrid (PAN6723) following inoculation with *Fusarium verticillioides* and infestation with *Busseola fusca* over three growing seasons

	Season		
Treatment	2008/09	2010/11	2011/12
Control	0.4 abc*	0.9 a-d	0.0 a
F. verticillioides	3.0 efg	2.2 cde	0.1 ab
F. verticillioides x *B. fusca*	5.2 h	0.9 a-d	2.0 a-d
B. fusca	1.3 a-e	0.9 a-d	2.3 c-f

$LSD_{(0.05)}$ = 2.1

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 2. *Busseola fusca* tunnel length (cm) in a non-*Bt* maize hybrid (PAN6723) following inoculation with *Fusarium verticillioides* and infestation with *Busseola fusca* over three growing seasons

	Season		
Treatment	2008/09	2010/11	2011/12
Control	2.1 cde*	0.6 abc	0.0 a
F. verticillioides	6.6 fg	0.0 a	0.1 ab
F. verticillioides x *B. fusca*	7.5 fg	0.0 a	1.1 ab
B. fusca	5.2 fg	0.0 a	0.8 a-d

$LSD_{(0.05)}$ = 1.8

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 3. Fumonisin production (µg/g) in a non-*Bt* maize hybrid (PAN6723) following inoculation with *Fusarium verticillioides* and infestation with *Busseola fusca* over three growing seasons

	Season		
Treatment	2008/09	2010/11	2011/12
Control	2.6 ab*	5.2 abc	0.1 a
F. verticillioides	22.9 f	11.3 de	2.2 ab
F. verticillioides x *B. fusca*	15.6 de	10.3 d	2.2 ab
B. fusca	5.2 abc	0.5 ab	2.0 ab

$LSD_{(0.05)}$ = 5.8

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 4. Seasonal effect on Fusarium ear rot development (%) in a non-*Bt* maize hybrid (PAN6723) to determine the effect of mechanical wounding to maize ears caused by cork borers

Season	Fusarium ear rot (%)
2009/10	9.7 c*
2010/11	6.4 b
2011/12	2.0 a

$LSD_{(0.05)}$ = 2.4

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 5. Treatment effect on Fusarium ear rot development (%) in a non-*Bt* maize hybrid (PAN6723) to determine the effect of mechanical wounding to maize ears caused by cork borers

Treatment	Fusarium ear rot (%)
Control	0.2 a*
F. verticillioides/1.59 cm single wound	1.9 ab
F. verticillioides/1.75 cm multiple wounds	6.0 bc
F. verticillioides/2.23 cm multiple wounds	6.0 bc
F. verticillioides/2.39 cm multiple wounds	11.2 de
1.59 cm single wound	2.0 ab
1.75 cm multiple wounds	7.1 cd
2.23 cm multiple wounds	8.2 cde
2.39 cm multiple wounds	11.9 e

$LSD_{(0.05)}$ = 4.2

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 6. Seasonal effect on fumonisin production (µg/g) on a non-*Bt* maize hybrid to determine the effect of mechanical wounding to maize ears caused by cork borers

Season	Fumonisin (µg/g)
2009/10	11.0 b*
2010/11	14.0 b
2011/12	5.4 a

$LSD_{(0.05)}$ = 3.1

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 7. Treatment effect on fumonisin levels (µg/g) in a non-*Bt* maize hybrid (PAN6723) to determine the effect of mechanical wounding to maize ears caused by cork borers

Treatment	Fumonisin (µg/g)
Control	2.5 a*
F. verticillioides/1.59 cm single wound	8.3 cd
F. verticillioides/1.75 cm multiple wounds	12.6 de
F. verticillioides/2.23 cm multiple wounds	14.7 e
F. verticillioides/2.39 cm multiple wounds	13.8 e
1.59 cm single wound	6.9 abc
1.75 cm multiple wounds	12.7 de
2.23 cm multiple wounds	9.6 cde
2.39 cm multiple wounds	9.9 cde

$LSD_{(0.05)}$ = 5.4

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 8. The effect of Season and Treatment on the mean (ng fungal DNA/0.5 mg milled sample) target DNA of fumonisin-producing *Fusarium* spp. in maize hybrid (PAN6723)

	Season		
Treatment	2009/10	2010/11	2011/12
Control	0.1 a*	0.2 ab	0.1 ab
F. verticillioides/1.59 cm single wound	0.5 abc	0.7 abc	0.1 ab
F. verticillioides/1.75 cm multiple wounds	3.5 f	1.5 abc	0.2 ab
F. verticillioides/2.23 cm multiple wounds	2.6 de	3.1 ef	0.2 ab
F. verticillioides/2.39 cm multiple wounds	3.5 f	3.7 f	0.4 abc
1.59 cm single wound	0.4 abc	0.5 abc	0.2 ab
1.75 cm multiple wounds	1.7 abc	1.8 abc	0.2 ab
2.23 cm multiple wounds	1.6 abc	2.5 de	0.4 abc
2.39 cm multiple wounds	1.8 abc	2.1 de	0.6 ab

$LSD_{(0.05)}$ = 1.2

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Figure 1. Typical Fusarium ear rot symptoms associated with *Busseola fusca* damage (second ear, left-right) and typical *B. fusca* damage which is not associated with ear rot (third ear, left-right).

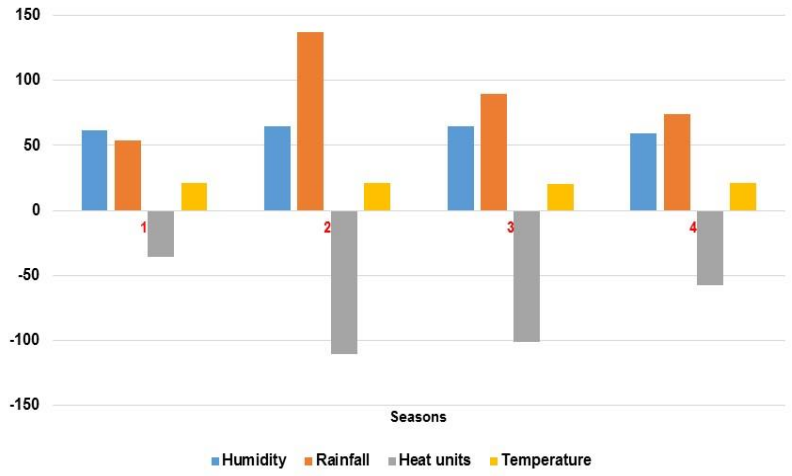

Figure 2. Mean rainfall (mm), relative humidity (%), heat units and temperature (˚C) during the 2008/09 (1), 2009/10 (2), 2010/11 (3) and 2011/12 (4) seasons in Potchefstroom obtained from ARC-ISCW.

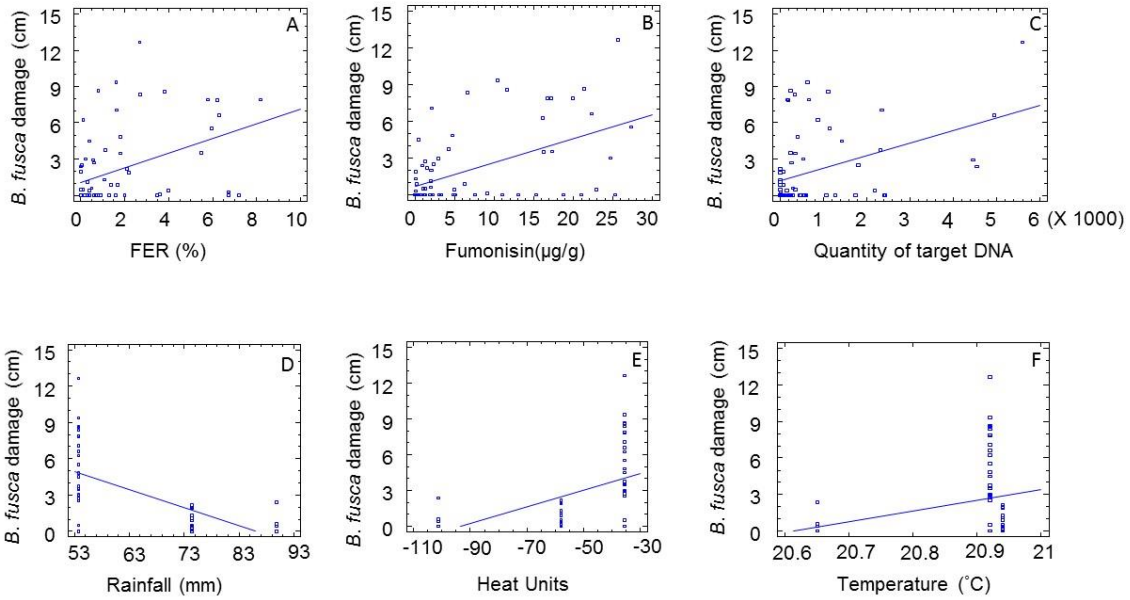

Figure 3. Larval damage to a maize hybrid (PAN6723) infested with *Busseola fusca* and inoculated with *Fusarium verticillioides*. Correlations, as determined by simple regression analysis, were between cumulative tunnel length caused by *Busseola fusca* damage and **(A)** Fusarium ear rot (FER; *P*-value = 0.000; *r* = 0.43; R^2 = 0.18; *n* = 72); **(B)** fumonisin production (*P*-value = 0.000; *r* = 0.52; R^2 = 0.27; *n* = 72); **(C)** quantity of target DNA of fumonisin-producing *Fusarium* spp. (ng fungal DNA/ 0.5 mg milled maize sample; *P*-value = 0.000; *r* = 0.41; R^2 = 0.17; *n* = 72); **(D)** mean rainfall (*P*-value = 0.000; *r* = -0.73; R^2 = 0.53; *n* = 72); **(E)** mean heat units (*P*-value = 0.000; *r* = 0.62; R^2 = 0.40; *n* = 72); and **(F)** mean temperature on a maize hybrid (PAN6723; *P*-value = 0.001; *r* = 0.39; R^2 = 0.15; *n* = 72).

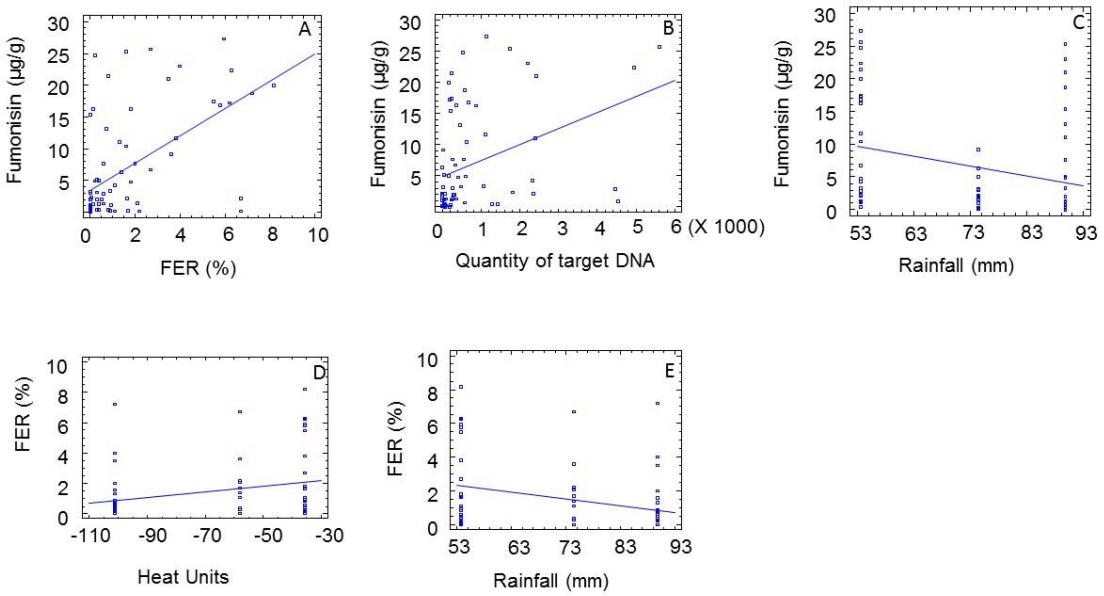

Figure 4. Fumonisin production and Fusarium ear rot (FER) development in a maize hybrid (PAN6723) infested with *Busseola fusca* larvae and inoculated with *Fusarium verticillioides*. Correlations, as determined by simple regression analysis, were between fumonisin production and **(A)** FER (*P*-value = 0.000; *r* = 0.56; R^2 = 0.32; *n* = 72); **(B)** quantity of target DNA of fumonisin-producing *Fusarium* spp. (ng fungal DNA/ 0.5 mg milled maize sample; *P*-value = 0.001; *r* = 0.38; R^2 = 0.14; *n* = 72) and **(C)** rainfall (*P*-value = 0.02; *r* = -0.27; R^2 = 0.08; *n* = 72). **(D)** Correlations, as determined by simple regression analysis, were between Fusarium ear rot (FER) and heat units (*P*-value = 0.04; *r* = 0.24; R^2 = 0.06; *n* = 72) and **(E)** mean rainfall (*P*-value = 0.02; *r* = -0.28; R^2 = 0.08; *n* = 72).

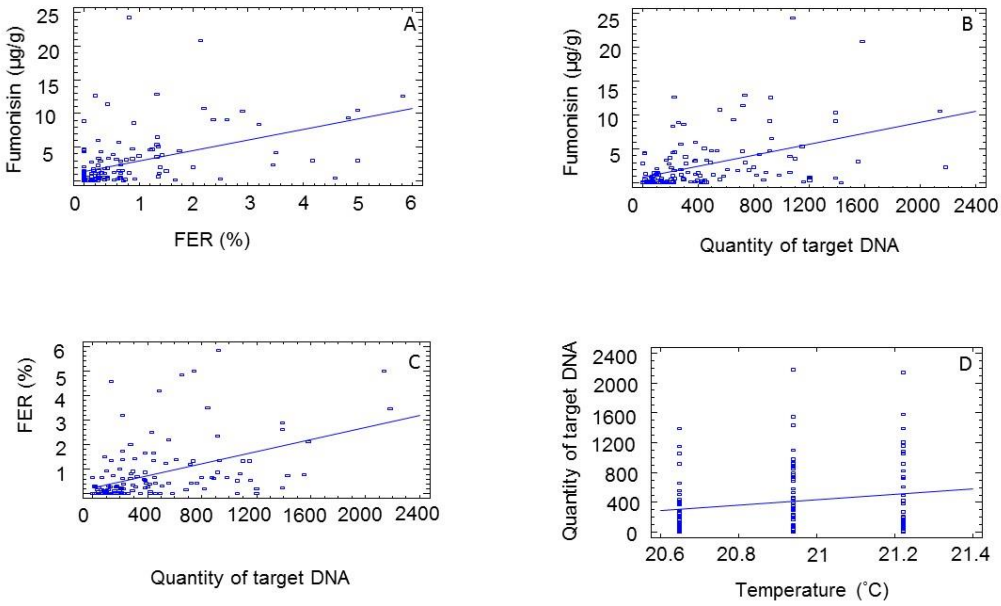

Figure 5. Fumonisin production, Fusarium ear rot (FER) development and fungal contamination of a maize hybrid (PAN6723) mechanically wounded with cork borers and inoculated with *Fusarium verticillioides*. Correlations, as determined by simple regression analysis, were between fumonisin production and **(A)** FER (*P*-value = 0.000; *r* = 0.47; R^2 = 0.22; *n* = 144) and **(B)** quantity of target DNA of fumonisin-producing *Fusarium* spp. (ng fungal DNA / 0.5 mg milled maize sample; *P*-value = 0.000; *r* = 0.48; R^2 = 0.21; *n* = 144). **(C)** Correlation, as determined by simple regression analysis, was between FER and quantity of target DNA of fumonisin-producing *Fusarium* spp. (*P*-value = 0.000; *r* = 0.48; R^2 = 0.23; *n* = 144). **(D)** Correlation, as determined by simple regression analysis, was between the quantity of target DNA of fumonisin-producing *Fusarium* spp. and mean temperature (*P*-value = 0.02; *r* = 0.19; R^2 = 0.04; *n* = 144).

CHAPTER 3

Fusarium ear rot and fumonisins in maize kernels when comparing a *Bt* hybrid with its non-*Bt* isohybrid and use of conventional insecticides to control *Busseola fusca* infestations

ABSTRACT

Maize production in South Africa is negatively affected by *Fusarium verticillioides*, an endophytic maize pathogen, as well as by *Busseola fusca* larval damage. *Fusarium verticillioides* causes ear, stem and root rot, and also produces fumonisin mycotoxins which are toxic to humans and livestock. *Busseola fusca* is a pest of economic importance that causes the most damage to maize plants in South Africa. In this study, the interaction between *F. verticillioides* and *B. fusca* was investigated to elucidate its effects on Fusarium ear rot (FER) and fumonisin production in a *Bt* hybrid (*MON810* gene) and its insect-susceptible non-*Bt* isohybrid. The effect of Beta-cyfluthrin (non-systemic, granular) and Benfuracarb (systemic, seed treatment) insecticide applications on FER and fumonisin production in maize was also determined in a conventional hybrid. For *B. fusca* infestations, larvae were dispensed into the whorl of each plant at the 12th leaf stage prior to tasselling, while a *F. verticillioides* MRC826 spore suspension was inoculated through the silks at the silking stage. Field trials were conducted over three seasons using a randomised complete block design with six replicates per treatment. Maize ears were harvested at physiological maturity and FER, total fumonisin levels, stem borer damage and target DNA of fumonisin-producing *Fusarium* spp. quantified. Significantly less FER and fumonisin were produced in the *Bt* maize hybrid compared to the non-*Bt* isohybrid under natural farming conditions, but fungal colonisation and fumonisin production under artificial *F. verticillioides* inoculation did not differ significantly between the *Bt* and non-*Bt* maize. Fumonisin production correlated moderately with the quantity of target DNA of fumonisin-producing *Fusarium* spp. Benfuracarb application resulted in a significant reduction in FER and fumonisin production while Beta-cyfluthrin did not. Moreover, *B. fusca* damage to maize ears significantly increased when both insecticides were not applied to the *B. fusca*-infested plants. This study has indicated that *Bt* maize and the Benfuracarb insecticide reduce *B. fusca* damage to maize ears, and are also indirectly effective in reducing FER and fumonisin production. However, this was not consistent over seasons due to differences in climatic conditions.

INTRODUCTION

Maize (*Zea mays* L.) is a summer crop that serves as staple food to millions of Africans, with average human consumption exceeding 300 g per person per day in rural areas of South Africa (Shephard *et al*., 2007). The crop is affected by the cosmopolitan fungus *Fusarium verticillioides* Sacc. Nirenberg (syn = *F. moniliforme* Sheldon), which occurs in many production regions of the world (Munkvold and Desjardins, 1997; Ncube *et al*., 2011). *Fusarium verticillioides* causes Fusarium root, stem and ear rots, with symptoms varying from non-symptomatic infections to severe rotting of infected plant parts (Munkvold *et al*., 1997). The most detrimental effect of *F. verticillioides*, however, is that it produces fumonisin mycotoxins that have been associated with diseases of humans and livestock (Marasas, 2001). Fumonisins are naturally occurring metabolites that are produced by *F. verticillioides* beginning from the early post-silking and the dough stages in maize kernels (Janse van Rensburg, 2012).

Busseola fusca (Fuller) (Lepidoptera: Noctuidae) is considered the most injurious pest of maize in South Africa (Van Rensburg and Flett, 2010). *Busseola fusca* is endemic in the Highveld and the western maize production regions of the country (Kfir and Bell, 1993; Kfir, 2000; 2002), and can cause an estimated annual loss of 10-60% of total maize production (Kfir *et al*., 2002) under favourable conditions such as, cool and humid climatic conditions (Van Rensburg *et al*., 1987a) at altitudes ranging from sea level to 2 000 m above sea level (Abate *et al*., 2000). *Busseola fusca* larvae feed mainly in the whorls of the plant until the fourth instar, after which they tunnel into the stem (Van Rensburg *et al*., 1989; Calatayud *et al*., 2014). Once inside the stem, the larvae cause extensive damage to internal stem tissue (Van Rensburg *et al*., 1988a) and a single larva usually feed per stem owing to the cannibalistic nature of *B. fusca* larvae (Robertson, 2000). *Busseola fusca* larvae also cause direct damage to maize ears, although this damage can be sporadic (Van Rensburg *et al*., 1988a). Wounds produced by lepidopteran insects provide a pathway for infection of maize ears and stems by airborne or rain-splashed *F. verticillioides* spores (Sobek and Munkvold, 1999). Maize pests of lesser importance in South Africa include *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) (Kfir *et al*., 2002) and *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) (Van den Berg, 1997; Van den Berg and Drinkwater, 2000).

Damage caused by *B. fusca* larvae can be reduced through the application of insecticides (Meissle *et al*., 2010; Beyene *et al*., 2011), crop residue management (Kfir *et al*., 2002), push-pull habitat manipulation (Khan *et al*., 2008) and biological control with parasitoids (Kfir *et al*., 2002). However, *B. fusca* damage is most effectively controlled by planting maize genetically modified with *Bacillus thuringiensis* (*Bt*) genes that encode for δ-endotoxin crystal proteins that are toxic to Lepidopteran insects (Hellmich *et al*., 2008; Ranum *et al*., 2014). *Bt* maize

hybrids have been found to be less prone to Fusarium ear rot (FER) and fumonisin contamination than non-*Bt* maize hybrids after infestation with *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) in Europe, north and south America (Munkvold *et al*., 1997; 1999; Bakan *et al*., 2002; Magg *et al*., 2002; Hammond *et al*., 2004; Barros *et al*., 2009). *Bt* hybrids also contain higher concentrations of lignin (Saxena and Stotzky, 2001; Poerschmann *et al*., 2005; Yanni *et al*., 2011), which improves the constitutive defence mechanisms in the plant (Freeman and Beattie, 2008). Lignin plays a central role in plant defence against insects and pathogens (Johnson *et al*., 2009; Barakat *et al*., 2010) and its synthesis is also induced by insect herbivory or pathogen attack (Ostrander and Coors, 1997; Barakat *et al*., 2010).

Bt maize comprising the *MON810* gene has been widely cultivated in South Africa to reduce *B. fusca* damage. Resistance in *B. fusca* larvae to the *MON810* gene has, however, occurred due to selection pressure derived from continuous exposure of larvae of the second seasonal moth flight to sub-lethal levels of the *Bt* toxin at late plant growth stages (Van Rensburg, 2007; Kruger *et al*., 2011; 2012). The *Bt*-protein concentration in silks of *Bt* maize (*MON810* gene) is considered low enough to allow survival of some larvae until completion of the first two instars, after which the ear tips and husk leaves serve as important feeding sites (Van Rensburg, 2001). Moreover, resistance to *Bt* maize is attributed to the compromised efficacy of the refuge strategy (Kruger *et al*., 2012). As a result, the maize industry recently introduced *Bt* plants containing the *MON89034* gene which produces the Cry1A.105 and Cry2Ab2 proteins (CERA, 2015). *MON89034* has a wider spectrum of insect control and is a much more effective insect resistance management tool (CERA, 2015).

In regions where national restrictions on *Bt* maize cultivation are enforced, insecticides are used to control insects such as stem borers (Meissle *et al*., 2010). The application of the pyrethroid insecticide Beta-cyfluthrin, a granular stomach and contact insecticide, has successfully controlled *B. fusca* and *C. partellus* infestations in Kenya (Beyene *et al*., 2011). A mixture of Endosulfan and Deltamethrin applied into the whorl or the sides of plants has also effectively controlled *B. fusca* in maize in South Africa (Van den Berg and Van Rensburg, 1996). The application of foliar insecticide spray formulations of Chlorpyrifos, Imidacloprid, Cypermethrin + Dimethoate and Lambda-cyhalothrin were further found to be effective in controlling *B. fusca* larvae in Nigeria (Adamu *et al*., 2015). Insecticide control can be economically applied during the vegetative growth stages when approximately 10% of plants are infected with *B. fusca* (Van Rensburg *et al*., 1988b). Application of insecticides in the whorl is potentially effective until shortly before tasselling, while application after tasselling or panicle appearance results in poor control of *B. fusca* because insecticides are ineffective against larvae in stems (Van Rensburg *et al*., 1987b). The cryptic feeding mechanism of *B. fusca* also has implications for control measures based on manual or mechanical insecticide applications. The effectiveness of insecticides such as Beta-cyfluthrin, however, can be enhanced by

targeting young larval instars, which are most vulnerable (Adamu *et al*., 2015), through scouting for stem borer eggs 2 weeks after plant emergence and applying the insecticide if eggs are present in over 5% of the plants. Benfuracarb is a systemic insecticide that provides residual protection against *B. fusca* stem borers over the entire pre-tasselling period (Van Rensburg *et al*., 1991) while Beta-cyfluthrin is a non-systemic insecticide for the control of *B. fusca* stem borers that is widely applied in subsistence farming systems in South Africa (Ncube, 2008).

Busseola fusca damage has been reported to increase the incidence of FER caused by *F. verticillioides* in conventional maize hybrids in South Africa (Flett and Van Rensburg, 1992). More than 70% of maize planted on commercial farms in South Africa is genetically modified for insect resistance and herbicide tolerance (James, 2012). Subsistence farmers, however, rely on insecticides; particularly Beta-cyfluthrin; to control *B. fusca* (Ncube, 2008). The effect of these *B. fusca* control strategies on FER and fumonisin production have not been determined in South Africa. This study, therefore, was conducted to elucidate the *F. verticillioides* and *B. fusca* interaction on FER and fumonisin production in a *Bt* hybrid and its non-*Bt* isohybrid over three seasons. FER and fumonisin production under conventional insecticide application in South Africa was also studied.

MATERIALS AND METHODS

Experimental design of trials

Field trials to evaluate the effect of *Bt* maize and insecticides on FER and fumonisin production in maize plants infested with *B. fusca* and inoculated with *F. verticillioides* were performed in the North West province of South Africa. A *Bt* trial, comprising of a commercial *Bt* maize hybrid (PAN6236B) expressing the *MON810* gene, and its insect-susceptible non-*Bt* isohybrid (PAN6126), was planted at the ARC-Grain Crops Institute (ARC-GCI) experimental farm in Potchefstroom (26°73'60.7"S; 27°07'55.3"E) during the 2009/10, 2010/11 and 2011/12 seasons. The *MON810* gene was the only commercially available *Bt* gene for the control of stem borers in maize in South Africa at the time of the study. The insecticide trial involved a non-*Bt* (conventional) maize hybrid (PAN6723), and was planted at the ARC-GCI experimental farm in Potchefstroom in October and late December 2012 (first and second trial, respectively), and at Buffelsvlei near Ventersdorp (26°49'38.6"S; 26°60'02.9"E) in November 2012. The insecticide trials were planted under conventional dry land conditions with hybrid seed purchased from Pannar Seeds, Greytown, South Africa. Soil analysis was performed before planting to calculate the quantity of fertilisers required, and pre-emergence (S-metolachlor) and post-emergence (thiadiazine) herbicides were applied for weed control according to the manufacturer's instructions for both field trials.

The treatments included in the *Bt* trial were *B. fusca* infestation only, *F. verticillioides* inoculation only, and *F. verticillioides* inoculation and *B. fusca* infestation combined. The control treatment was neither infested with *B. fusca* nor inoculated with *F. verticillioides*. Treatments for the insecticide trial included: *F. verticillioides* x *B. fusca* x Beta-cyfluthrin, *F. verticillioides* x *B. fusca* x Benfuracarb, *F. verticillioides* x *B. fusca*, *F. verticillioides* only, *B. fusca* only, *B. fusca* x Beta-cyfluthrin, *B. fusca* x Benfuracarb and a control treatment that was neither treated with insecticides nor inoculated with *F. verticillioides* and/or infested with *B. fusca.* The Benfuracarb; a systemic pesticide; was applied to maize seed prior to planting according to the manufacturer's instructions, while Beta-cyfluthrin; a non-systemic pesticide; was applied directly into the whorl according to the manufacturer's instructions, weekly, beginning 2 weeks after emergence until tasselling. All field trials consisted of six replicates planted in a randomised complete block design. The experimental row was bordered by two rows on each side to manage inter-row interference. The rows were 5 m in length, with an intra-row spacing of 30 cm and inter-row spacing of 1.5 m. All primary maize ears in each experimental row were hand harvested at physiological maturity.

***Fusarium verticillioides* inoculation and *B. fusca* infestation**

Fungal inoculum was prepared by culturing a high fumonisin-producing *F. verticillioides* isolate MRC826 (Gelderblom *et al*., 2001), obtained from the Medical Research Council (MRC) at Tygerberg in South Africa, on potato dextrose agar (PDA) for 4 days at 25˚C. Two agar plugs from the actively growing culture were then used to inoculate 100 ml sterile Armstrong *Fusarium* medium (Booth, 1971) in 200-ml Erlenmeyer flasks, followed by incubation on a rotary shaker at 100 revolutions per min (rpm) at 25˚C. After 4 days, the *F. verticillioides* spore suspension was filtered through two layers of sterile cheesecloth into a 250-ml, wide-mouth centrifuge bottle (Nalgene, Lasec, Johannesburg, South Africa). The suspension was then spun at 1 000 rpm using a swinging bucket rotor in a Hermle Z400® centrifuge (Hermle Labortechnik, Wehingen, Germany) for 10 min, and the supernatant was decanted. The *Fusarium* medium was removed by washing spores twice in 100 ml sterile distilled water that was previously de-ionised using a PureLab Ultra® machine (Elga Process Water, Marlow, UK).

The *F. verticillioides* spore suspension was diluted to 2×10^6 spores ml^{-1} using a Fuchs Rosenthal haemocytometer (Hawksley, London, UK) and Axioskop® Routine microscope (Carl Zeiss, Oberkochen, Germany). Tween 20 surfactant (Fischer Biotech, Fairlawn, NJ, USA) was then added to the spore suspension at a rate of 30 µl L^{-1} to minimise the clumping of spores. Maize ears were inoculated with *F. verticillioides* by injecting 2 ml of the *F. verticillioides* conidial spore suspension into the silk channel of each primary ear at the silking stage, using a cattle injector fitted with an 18 G × 1.5-in. (1.20 × 38 mm) Terumo needle (sterile, nontoxic, and nonpyrogenic) (Senwes, Potchefstroom), as described by Small *et al*. (2012).

Neonate larvae of *B. fusca* were produced at the mass rearing facility at the ARC-GCI in Potchefstroom (Van Rensburg and Van Rensburg, 1993). Aliquots of 10-15 neonate larvae were deposited into the whorl of each plant at the 12th leaf stage before tasselling using a mechanical applicator that was calibrated to dispense between 10-15 neonate larvae (Van Rensburg and Van Rensburg, 1993). Inoculation at this stage facilitates that larvae will also migrate to ears of plants and not migrate directly to stems, as is the case with early borer infestations (J.B.J. van Rensburg, personal communication).

Field trial evaluation

Fusarium ear rot and B. fusca *damage*

FER symptoms on each ear were visually rated as described by Ennerson and Hunter (1980). FER is visible as pink or white mycelial growth on damaged kernels (White, 1999) and can also appear as pink or streaked kernel discolouration without kernel damage. The discoloured area was expressed as a percentage of the total surface. FER ratings excluded tunnels that did not show visible FER symptoms. However, damaged kernels in tunnels with visible FER symptoms were included in the FER ratings. Each ear from each experimental row (replicate) was rated individually, after which an average FER score for each experimental row (replicate) was calculated (Chapter 2). *Busseola fusca* damage was determined by measuring the cumulative feeding tunnel length (cm) on each ear (Chery *et al*., 2004) upon harvest. Each ear was first rated individually, and the mean *B. fusca* damage was thereafter calculated for each experimental row.

Quantification of fumonisin-producing Fusarium *spp.*

The quantity of target DNA of fumonisin-producing *Fusarium* spp. in 0.5-mg sample of all milled maize kernels from each replicate was determined using quantitative real-time PCR (qPCR) (Chapter 2). A DNeasy Plant Mini Kit® (Qiagen, Venlo, Netherlands) was used to extract fungal DNA, and all DNA samples were then measured on a NanoDrop 2000c spectrophotometer (NanoDrop, Wilmington, DE, USA) and diluted to a concentration of 10 ng with molecular grade water (Melford Laboratories, Ipswich, UK). The extracted DNA was thereafter frozen at -20˚C until analysis.

Clear, low profile 96-well PCR plates (Bio-Rad Laboratories, Hercules, CA, USA) were used to perform qPCR analysis. Assays were performed in a 25-µl total reaction volume consisting of 4 µl of sample DNA and 21 µl master mix: 2.125 µl *FUM1*- probe (1 µM), 0.875 µl Taqfum-2F forward primer (0.33 µM), 0.875 µl Vpgen-3R reverse primer (0.33 µM), 12.5 µl Sensimix® (Bioline, London, UK) and 4.625 µl molecular grade water (Melford Laboratories). The probe and primers used were designed by Waalwijk *et al*. (2008). *Fusarium verticillioides* MRC826 was used as reference culture to prepare five DNA standards with concentrations of

10 ng, 1 ng, 100 pg, 10 pg and 1 pg to construct a standard curve. The qPCR reaction was performed on a CFX96™ Real-Time System (Bio-Rad Laboratories) using the following cycling conditions: 95˚C for 10 min, followed by 40 PCR cycles at 95˚C for 10 s, 60˚C for 30 s and 72˚C for 10 s. An iCycler™ iQ Optical System Software Version 3.0a (Bio-Rad Laboratories) was used to quantify target DNA of fumonisin-producing *Fusarium* spp. The efficiency of the qPCR runs ranged from 96.1-99.9%, while the R^2 ranged from 0.992-0.997.

Fumonisin production

Fumonisin analysis was performed at the ARC-GCI Mycotoxin Laboratory, Potchefstroom, using High-Performance Liquid Chromatography (HPLC) (Waters Corp., Milford, MA, USA). The FumoniTest™ HPLC procedure (Vicam®, Watertown, MA, USA) was performed on a 50-g sub-sample taken from the 250-g maize kernel samples from all threshed kernels from each replicate. The samples were all ground to a powder with a Cyclotec sample mill (Foss Tecator, Hoganas, Sweden) with a 1-mm mesh sieve after shelling.

Individual concentrations of fumonisin B_1, B_2 and B_3 were quantified by the FumoniTest™ HPLC method (Vicam®). *O*-phthaldialdehyde was used to derivatise fumonisins, followed by quantification using an HPLC Symmetry® C18 column (Waters Corp.) with dimensions of 3.9 × 150 mm and a volume of 4 µl. The mobile phase consisted of methanol (Romil, Cambridge, UK) : 0.1 M NaH_2PO_4 (Merck, Johannesburg, South Africa) (77:23, v/v), adjusted to a pH of 3.3 with *o*-phosphoric acid (Merck). The flow rate was 0.8 ml/min. A scanning fluorescence detector (Waters® 474) with an excitation of 335 nm and emission of 440 nm was used. A multi λ fluorescence detector and Breeze™ software (Waters Corp.) were part of the HPLC system. Retention times for FB_1, FB_2 and FB_3 were approximately 5.5, 11.5 and 12.5 min, respectively. Five fumonisin standards (MRC) containing a total fumonisin (B_1, B_2 and B_3) concentration of 2, 5, 10, 15 and 20 µg/g, respectively, were included in each run.

Meteorological data

Rainfall, heat units, humidity and temperature data for the 2009/10-2011/12 seasons for Potchefstroom was obtained from the Agricultural Research Council-Institute of Soil, Climate and Water (ARC-ISCW), Pretoria, South Africa on a mean monthly basis between January and April. The period January - April is important in the FER and fumonisin context because it covers the period between silking and the dent stage at which FER and fumonisin production in maize takes place (Murillo-Williams and Munkvold, 2008; Janse van Rensburg, 2012). Heat units quantifying the thermal environment of crops (Brown, 2013) were obtained from ARC-ISCW.

Statistical analyses

An analysis of variance (ANOVA) was performed on the data for FER, fumonisin production, quantity of target DNA of fumonisin-producing *Fusarium* spp. and the cumulative tunnel length caused by *B. fusca* damage (*B. fusca* damage) by using GenStat 14th edition (VSN, International, Hemel Hempstead, UK) at Tukey's 95% confidence interval.

Simple regression analyses between fumonisin production and FER, and quantity of target DNA of fumonisin-producing *Fusarium* spp.; as well as between FER and quantity of target DNA of fumonisin-producing *Fusarium* spp. were performed using Statgraphics® 5 Plus (Statpoint Technologies Incorporated, Warrenton, VA, US) across three seasons on a *Bt* hybrid (PAN6236B).

In the non-*Bt* maize isohybrid (PAN6126), simple regression analyses between *B. fusca* damage and FER, mean rainfall, and mean heat units; as well as between fumonisin production and quantity of target DNA of fumonisin-producing *Fusarium* spp. were performed using Statgraphics® 5 Plus (Statpoint Technologies) across three seasons.

In the insecticide-treated trial, simple regression analyses between *B. fusca* damage and FER, mean fumonisin production and quantity of target DNA of fumonisin-producing *Fusarium* spp.; between fumonisin production and FER, and quantity of target DNA of fumonisin-producing *Fusarium* spp.; as well as between FER and quantity of target DNA of fumonisin-producing *Fusarium* spp. were performed using Statgraphics® 5 Plus (Statpoint Technologies) across three seasons.

RESULTS

The effect of *Bt* maize on *F. verticillioides* infection and insect damage

Fusarium ear rot and B. fusca *damage*

FER development in *Bt* and non-*Bt* maize ($P<0.05$) was significantly affected by hybrid, season (Table 1) and treatment effects (Table 2). Significantly less FER developed in the *Bt* hybrid than in its non-*Bt* isohybrid during the 2010/11 and 2011/12 seasons, but not in the 2009/10 season where there was no significant difference in FER between the *Bt* hybrid and non-*Bt* isohybrid (Table 1). Moreover, there were no significant differences in FER in the *Bt* hybrid across seasons (Table 1). *Busseola fusca* infestation, alone and in combination with *F. verticillioides*, was associated with significantly more FER in the non-*Bt* isohybrid than in the *Bt* hybrid (Table 2). None of the treatments significantly affected FER development in the *Bt* hybrid. Ear damage, caused by *B. fusca* in the insect-susceptible non-*Bt* isohybrid during the 2011/12 season, was significantly more ($P<0.05$) than in the *Bt* hybrid, and in the other growing seasons (Table 3).

Quantification of fumonisin-producing Fusarium *spp.*

Mean quantity of target DNA of fumonisin-producing *Fusarium* spp. in kernels of *Bt* and non-*Bt* maize (P<0.05) are presented across three seasons (Table 4). In the 2009/10 season, significantly more target DNA was found in ears inoculated with *F. verticillioides*, with or without *B. fusca* infestation, in both the *Bt* hybrid and the non-*Bt* hybrid (Table 4). In the 2011/12 season, however, inoculation with *F. verticillioides* did not significantly differ from *B. fusca* infestation alone, but differed from the control. *Busseola fusca* infestation was not associated with higher fungal colonisation of maize ears. There was no significant difference in the quantity of target DNA of fumonisin-producing *Fusarium* spp. between the *Bt* hybrid and the non-*Bt* isohybrid. Moreover, there were no significant differences across treatments in the 2010/11 season.

Fumonisin production

Fumonisin production was assessed by season, with inoculation treatments (Table 5), and comparing *Bt* and non-*Bt* hybrids (Table 6). Significantly (P<0.05) more fumonisins were produced in maize ears inoculated with *F. verticillioides*, with and without *B. fusca* in the 2009/10 and 2010/11 seasons, but not in the 2011/12 season. There were, however, no significant differences in fumonisins produced in maize kernels inoculated with *F. verticillioides* alone in the 2010/11 and 2011/12 seasons, but fumonisin production was significantly higher in the 2009/10 season. The *B. fusca* treatment in the 2011/12 season was associated with higher fumonisin levels than the control of the same season (Table 5), but this did not happen in the other seasons. Incidentally, significant *B. fusca* damage occurred in the non-*Bt* isohybrid during the 2011/12 season (Table 3). Across years, maize ears inoculated with *F. verticillioides* with and without *B. fusca* infestation were significantly more contaminated with fumonisin than in the control and the *B. fusca*-infested treatment in the *Bt* hybrid (Table 6). The control treatment of the *Bt* hybrid had significantly lower fumonisin levels than the non-*Bt* hybrid control. *Busseola fusca* infestation alone was not significantly associated with fumonisin production in either of the hybrids (Table 6). Fumonisin production under artificial *F. verticillioides* inoculation did not differ significantly between the *Bt* hybrid and its non-*Bt* isohybrids (Table 6).

The effect of insecticides on *F. verticillioides* and *B. fusca* damage of maize

FER and fumonisin production in treatments with *F. verticillioides* and *B. fusca* were lower with Benfuracarb, possibly due to reduced stem borer damage. Benfuracarb application consistently reduced FER (Table 7) and fumonisin production (Table 8) significantly (P<0.05) in a conventional hybrid inoculated with *F. verticillioides* and infested with *B. fusca* when compared to Beta-cyfluthrin. *Busseola fusca* damage significantly increased in the treatment

that was only infested with *B. fusca* and also in the combined *F. verticillioides*-inoculated and *B. fusca*-infested treatment when compared to the control in the absence of both insecticides. Benfuracarb reduced *B. fusca* damage while Beta-cyfluthrin did not (Table 9).

Meteorological data

The trials in this study were planted during the 2009/10, 2010/11 and 2011/12 seasons. The highest heat units and the lowest rainfall from the period January - April were recorded in the 2011/12 season followed by the 2010/11 season (Fig. 1). The 2009/10 season had the highest rainfall and lowest heat units (Fig. 1). No hail damage to maize was recorded in Potchefstroom during this study.

Simple regression analyses

A positive correlation was obtained between fumonisin production and FER (r = 0.39), and quantity of target DNA of fumonisin-producing *Fusarium* spp. (r = 0.63); and between FER and quantity of target DNA of fumonisin-producing *Fusarium* spp. in the *Bt* hybrid (r = 0.3) (Table 10). In the non-*Bt* hybrid, the cumulative tunnel length caused by *B. fusca* damage (*B. fusca* damage) poorly correlated with FER (r = 0.23). Moreover, *B. fusca* damage had a moderate negative correlation with mean rainfall (r = -0.46) and a moderate positive correlation with mean heat units (r = 0.50). A positive correlation (r = 0.56) was obtained between fumonisin production and quantity of target DNA of fumonisin-producing *Fusarium* spp. (Table 10).

In the insecticide-treated trial, poor correlations were obtained between *B. fusca* damage and FER (r = 0.28), mean fumonisin production (r = 0.23), and quantity of target DNA of fumonisin-producing *Fusarium* spp. (r = 0.17). Moderate correlations between fumonisin production and FER (r = 0.47), and quantity of target DNA of fumonisin-producing *Fusarium* spp. (r = 0.46); and between FER and quantity of target DNA of fumonisin-producing *Fusarium* spp. (r = 0.48) were obtained (Table 10).

DISCUSSION

This study demonstrated that *Bt* maize and Benfuracarb have a significant impact on *B. fusca* damage, and under some environmental conditions will be associated with less FER development in maize in South Africa. This reduction can be attributed to a reduction in stem borer damage in *Bt* maize compared to non-*Bt* maize, particularly during the 2011/12 season. Stem borers have been reported to be associated with increased FER before (Flett and Van Rensburg, 1992; Munkvold *et al*., 1997; Bakan *et al*., 2002), and this appeared to be due to the damage they caused to maize kernels that stimulate fungal contamination and the

discolouration of maize kernels. Wounds created by *B. fusca* can enable infection of maize plants with *F. verticillioides* resulting in the transition from symptomless endophtytism to necrotrophic pathogenicity (Rutherford *et al*., 2002). On the other hand, Ako *et al*. (2003) showed that lepidopteran pests are significantly attracted to maize that is infected with *F. verticillioides*. Therefore, it is likely that the damage caused by the two agents is synergistic. What this study did not demonstrate, however, was that *B. fusca* infestation had an effect on fungal colonization and fumonisin production. Both *Bt* and non-*Bt* maize kernels were equally contaminated with fumonisin-producing *Fusarium* spp., indicating that *Bt* maize does not have an effect on colonisation of maize ears with fumonisin-producing *Fusarium* spp. The positive correlation found between fungal colonization and fumonisin production also suggested that *B. fusca* infestation would not have a significant effect on fumonisin production. Moreover, fumonisin production by *F. verticillioides* was independent of *B. fusca* damage.

Busseola fusca infestation did not result in a significant increase in ear damage in the *Bt* hybrid thereby indicating the effectiveness of the *Bt* toxin against *B. fusca* larvae. The control treatment of the *Bt* hybrid had significantly lower fumonisin levels than that of the non-*Bt* isohybrid, indicating that *Bt* hybrids can be used to manage fumonisin production in maize under natural South African farming conditions. However, there was no difference in fumonisin production in both the *Bt* hybrid and its non-*Bt* isohybrid under artificial *F. verticillioides* inoculation in the absence of *B. fusca* infestation. This also indicate that the *Bt* hybrid has no effect on colonisation of maize ears with fumonisin-producing *Fusarium* spp. and subsequent production of fumonisins. Therefore, the effect of *Bt* maize on fumonisin production in maize ears was indirectly associated with its control of stem borer damage.

Benfuracarb application significantly reduced the levels of FER and fumonisin production while Beta-cyfluthrin did not, however this was confounded, since fumonisin production and FER are highly correlated. This is possibly due to the residual protection against *B. fusca* that was contributed by Benfuracarb over the entire pre-tasselling period (Van Rensburg *et al*., 1991). Benfuracarb also has a dual role as it can control root-lesion nematodes *Pratylenchus zeae* Graham and *P. brachyurus* Godfrey 9 weeks after maize planting in South Africa (McDonald *et al*., 1987). This results in the reduction in nematode wounding that might promote *F. verticillioides* infection through wounded lateral roots. The most likely way for infection with *F. verticillioides* is, however, through the silk channel (Munkvold and Carlton, 1997; Galperin *et al*., 2003) and wounds on maize ears (Chapter 2).

Beta-cyfluthrin was applied in the whorls and was not as effective as Benfuracarb in reaching stem borers in all plant tissues. *Busseola fusca* damage to maize ears significantly increased compared to the control when both insecticides were not applied to the *B. fusca*-infested treatments, indicating the effectiveness of both insecticides in the control of *B. fusca* larvae. This study further showed that *B. fusca* infestation, as in *Bt* maize, also had no effect

on the quantity of target DNA of fumonisin-producing *Fusarium* spp. However, *Bt* maize under natural *F. verticillioides* infection and maize plants treated with Benfuracarb resulted in a significant reduction in fumonisin levels. This is in agreement with a study by Folcher *et al*. (2009) who indicated that the application of an insecticide, Deltamethrin, controlled *O. nubilalis* and *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae) but did not affect the *Fusarium* mycoflora. The insecticide, however, also resulted in the reduction in fumonisin production (Folcher *et al*., 2009).

Despite the evidence found in this study and in others that *Bt* maize reduces FER, this was not consistent over seasons. In the 2009/10 season, for instance, no significant reduction in FER was found between *Bt* and non-*Bt* maize. Moreover, FER development was significantly lower than in the 2010/11 and 2011/12 seasons in the non-*Bt* isohybrid. The low FER incidence in 2009/10 might be due to cool and wet conditions, which are not favourable for disease development (Murillo-Williams and Munkvold, 2008; Janse van Rensburg, 2012). *Busseola fusca* damage has also been significantly negatively correlated with rainfall and positively correlated with higher temperatures (Van Rensburg *et al*., 1987a; Ebenebe *et al*., 2000), making this year unfavourable for the pest.

FER development and fumonisin production by *F. verticillioides* inoculum varied over seasons, indicating the importance of environmental conditions on FER and fumonisin production. Moreover, *B. fusca* infestation was not associated with an increase in fumonisin production in the 2009/10 and 2010/11 seasons, while it was in the 2011/12 season. Incidentally, it was during the 2011/12 season where *B. fusca* infestation resulted in a significant increase in ear damage in the non-*Bt* isohybrid. This suggests that an increase in the severity of damage to maize ears is important for fumonisin production (Chapter 2). This study has indicated that *Bt* maize and Benfuracarb insecticide reduced *B. fusca* damage to maize ears and were, therefore, indirectly effective for reducing FER and fumonisin production. This further suggest that Benfuracarb application is a good management strategy when *B. fusca* and *F. verticillioides* are likely to be co-occurring, particularly in South African maize farming context, where commercial maize production is largely reliant on *Bt* maize for the control of stem borers (James, 2012; Van den Berg, 2012). Seasonal variation in FER and fumonisin production by *F. verticillioides*, however, indicated the importance of climatic conditions on FER and fumonisin production in maize.

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Table 1. Fusarium ear rot (%) in a *Bt* (PAN6236B) and its non-*Bt* (PAN6126) maize isohybrid from field trials in three seasons

	Season		
Hybrid	2009/10	2010/11	2011/12
Bt	3.6 a-d*	3.2 abc	1.8 a
non-*Bt*	4.0 a-e	8.5 g	6.1 ef

$LSD_{(0.05)}$ = 2.2

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 2. Mean Fusarium ear rot (%) across three seasons in a *Bt* (PAN6236B) and its non-*Bt* (PAN6126) maize isohybrid when inoculated with *F. verticillioides* and/or *B. fusca* compared to a non-inoculated control

	Hybrid	
Treatment	*Bt*	non-*Bt*
Control	2.3 a*	3.3 a-e
F. verticillioides	3.3 a-e	4.5 a-f
F. verticillioides x *B. fusca*	3.1 a-d	8.2 g
B. fusca	2.7 abc	8.7 g

$LSD_{(0.05)}$ = 2.6

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 3. Impact of season and inoculation treatment on tunnel length (cm) in a *Bt* (PAN6236B) and its non-*Bt* (PAN6126) maize isohybrid

		Season		
Cultivar	Treatment	2009/10	2010/11	2011/12
Bt	Control	0.1 a*	0.0 a	0.1 a
	F. verticillioides	0.3 a	0.1 a	0.0 a
	F. verticillioides x *B. fusca*	0.0 a	0.2 a	0.0 a
	B. fusca	0.0 a	0.0 a	0.0 a
non-*Bt*	Control	0.0 a	0.3 a	0.0 a
	F. verticillioides	0.2 a	0.4 a	0.0 a
	F. verticillioides x *B. fusca*	0.3 a	0.4 a	3.3 b
	B. fusca	0.0 a	0.0 a	3.6 b

$LSD_{(0.05)}$ = 0.7

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 4. Mean (ng fungal DNA / 0.5 mg milled maize sample) DNA of fumonisin-producing *Fusarium* spp. in both the *Bt* (PAN6236B) hybrid and its non-*Bt* (PAN6126) maize isohybrid comparing inoculation treatments and control across three seasons

	Season		
Treatment	2009/10	2010/11	2011/12
Control	1.2 ab*	1.3 ab	0.2 a
F. verticillioides	4.4 d	2.0 b	2.2 bc
F. verticillioides x *B. fusca*	2.5 c	1.6 b	2.2 bc
B. fusca	0.8 ab	1.2 ab	0.7 ab

$LSD_{(0.05)}$ = 1.3

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 5. Impact of season and inoculation treatment on fumonisin production (µg/g) in a *Bt* (PAN6236B) and its non-*Bt* (PAN6126) maize isohybrid

	Season		
Treatment	2009/10	2010/11	2011/12
Control	6.0 ab*	6.3 ab	3.6 a
F. verticillioides	30.3 e	13.9 cd	9.8 abc
F. verticillioides x *B. fusca*	19.6 d	13.4 cd	9.4 abc
B. fusca	7.8 abc	6.4 ab	10.5 bc

$LSD_{(0.05)}$ = 6.6

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 6. Mean fumonisin production (µg/g) across three seasons in a *Bt* hybrid (PAN6236B) and its non-*Bt* (PAN6126) maize isohybrid

	Cultivar	
Treatment	*Bt*	Non-*Bt*
Control	2.2 ab*	8.4 cd
F. verticillioides	18.9 e	17.0 e
F. verticillioides x *B. fusca*	16.8 e	11.4 cd
B. fusca	5.6 abc	10.8 cd

$LSD_{(0.05)}$ = 5.3

*Means bearing the same letter(s) are not significantly different using Tukey's 95% confidence interval

Table 7. Mean Fusarium ear rot (%) development on ears of a conventional non-*Bt* maize hybrid (PAN6723) that were treated with Benfuracarb and Beta-cyfluthrin

Treatment	FER (%)
Control	0.23 a*
F. verticillioides x *B. fusca* x Beta-cyfluthrin	0.93 ab
F. verticillioides x *B. fusca* x Benfuracarb	0.53 a
F. verticillioides x *B. fusca*	1.72 b
F. verticillioides	0.8 ab
B. fusca	1.02 ab
B. fusca x Beta-cyfluthrin	0.61 a
B. fusca x Benfuracarb	0.13 a

$LSD_{(0.05)}$ = 1.1

* Means bearing the same letter are not significantly different using Tukey's 95% confidence interval.

Table 8. Mean fumonisin production (µg/g) on ears of a conventional non-*Bt* maize hybrid (PAN6723) that were treated with Benfuracarb and Beta-cyfluthrin

Treatment	Fumonisin (µg/g)
Control	1.01 a*
F. verticillioides x *B. fusca* x Beta-cyfluthrin	3.72 ab
F. verticillioides x *B. fusca* x Benfuracarb	1.04 a
F. verticillioides x *B. fusca*	5.40 b
F. verticillioides	3.05 ab
B. fusca	3.22 ab
B. fusca x Beta-cyfluthrin	2.32 ab
B. fusca x Benfuracarb	0.42 a

$LSD_{(0.05)}$ = 2.4

* Means bearing the same letter are not significantly different using Tukey's 95% confidence interval.

Table 9. Mean *Busseola fusca* damage (cm) on ears of a conventional non-*Bt* maize hybrid (PAN6723) that were treated with Benfuracarb and Beta-cyfluthrin

Treatment	Tunnel length (cm)
Control	0.1 a*
F. verticillioides x *B. fusca* x Beta-cyfluthrin	0.15 ab
F. verticillioides x *B. fusca* x Benfuracarb	0.26 ab
F. verticillioides x *B. fusca*	0.54 b
F. verticillioides	0.05 a
B. fusca	0.42 b
B. fusca x Beta-cyfluthrin	0.22 ab
B. fusca x Benfuracarb	0.11 a

$LSD_{(0.05)}$ = 0.3

* Means bearing the same letter are not significantly different using Tukey's 95% confidence interval.

Table 10. Regression analyses in a *Bt* (PAN6236B), its non-*Bt* (PAN6126) isohybrid and an unrelated non-*Bt* maize hybrid (PAN6723) that was treated with Benfuracarb and Beta-cyfluthrin insecticides from field trials to determine the effect of the *Busseola fusca* x *Fusarium verticillioides* interaction in maize

	Bt hybrid			non-*Bt* isohybrid			Conventional hybrid (Insecticide)		
	r	R^2	*P*-value	*r*	R^2	*P*-value	*r*	R^2	*P*-value
B. fusca x FER			ns***	0.23	0.05	0.05	0.28	0.08	0.00
B. fusca x fumonisin			ns			ns	0.23	0.05	0.01
B. fusca x DNA*			ns			ns	0.17	0.03	0.04
B. fusca x Rainfall			ns	-0.46	0.21	0.00			ns
B. fusca x HU**			ns	0.5	0.25	0.00			ns
B. fusca x Temperature			ns			ns			ns
B. fusca x Humidity			ns			ns			ns
Fumonisin x FER	0.39	0.15	0.00			ns	0.47	0.22	0.00
Fumonisin x DNA	0.63	0.40	0.00	0.56	0.31	0.00	0.46	0.21	0.00
FER x DNA	0.30	0.09	0.01			ns	0.48	0.23	0.00

DNA*, target DNA of fumonisin producing *Fusarium* spp.; HU**, Heat units, ns***, not significant

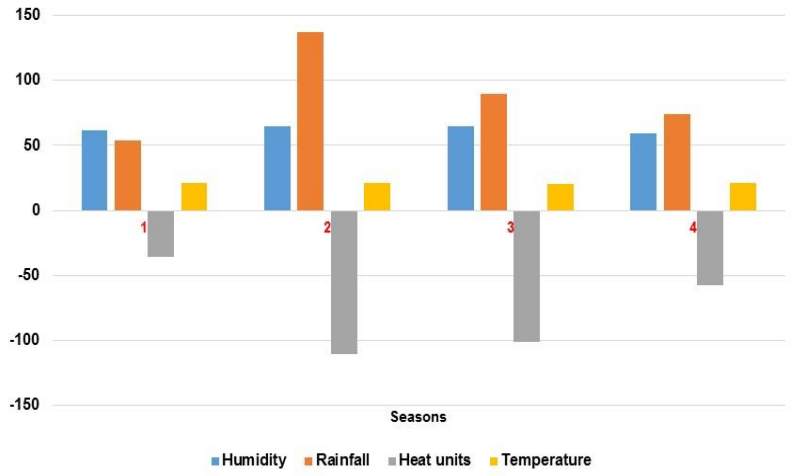

Figure 1. Mean rainfall (mm), relative humidity (%), heat units and temperature (˚C) during the 2008/09 (1), 2009/10 (2), 2010/11 (3) and 2011/12 (4) seasons in Potchefstroom (ARC-ISCW, 2016).

CHAPTER 4

Mycoflora of *Busseola fusca* frass, and the possible role of larvae in disseminating *Fusarium verticillioides*

ABSTRACT

Busseola fusca and *Fusarium verticillioides* are considered the most important pest and pathogen of maize in South Africa, respectively. *Busseola fusca* tunnels into stems and ears of maize plants, whereas *F. verticillioides* causes Fusarium ear rot and deposits fumonisins in maize kernels. Frass (excreta) deposited by *B. fusca* larvae in maize stems and ears during feeding could potentially be contaminated with pathogens such as *F. verticillioides*, thereby indirectly and passively contributing to plant diseases. The mycoflora present in frass of *B. fusca* larvae was thus investigated in this study, and the dissemination of fumonisin-producing *Fusarium* spp. by *B. fusca* larvae in maize stems determined. *Busseola fusca* frass was collected from plants with visible insect damage in three maize-growing districts in South Africa. The mycoflora in the frass was isolated and identified morphologically to genus level, followed by the sequencing of target genes for species identification. Species of *Acremonium, Aspergillus, Fusarium, Mucor, Rhizopus* and *Talaromyces* were associated with the *B. fusca* frass. The role of *B. fusca* larvae in disseminating fumonisin-producing *Fusarium* spp. was then determined in greenhouse and field trials. Maize whorls were inoculated with *F. verticillioides* MRC826 spores 4 weeks after plant emergence, and infested with *B. fusca* larvae 2 days later. The stems were split open after 3 weeks and frass collected from feeding channels, thereafter, target DNA of fumonisin-producing *Fusarium* spp. was quantified using real-time PCR. Target DNA of fumonisin-producing *Fusarium* spp. was significantly higher in frass collected from greenhouse plants inoculated with *F. verticillioides* than in frass collected from the uninoculated control, indicating that the inoculation was successful in the absence of soil-borne inoculum. Nevertheless, the field trial showed no significant differences in target DNA in frass from inoculated and non-inoculated plants. This is possibly due to natural *F. verticillioides* infection of maize plants in the field. This study further indicated that *B. fusca* frass also contained maize pathogens such as *Aspergillus* spp. and *Fusarium* spp., which could cause ear rot diseases. The occurrence of *Acremonium zeae* in frass has potential implications for the biological control of *F. verticillioides*, as the fungus produces pyrrocidines A and B antibiotics which are known to be antagonistic to *F. verticillioides*. The occurrence of *Aspergillus niger* in frass requires further investigation in order to clarify its role in fumonisin contamination of maize in South Africa.

INTRODUCTION

Maize (*Zea mays* L.) is the most important food crop grown in South Africa. From 2010-2016 the country has produced an average of 10.8 million tons of maize annually, with a range of 7.5-14.3 million tons per season (CEC, 2016). Approximately 9 million tons is locally consumed as food and feed, and the surplus is exported (DAFF, 2014). Maize production in the country, however, is hampered by *Fusarium verticillioides* Sacc. Nirenberg (syn = *F. moniliforme* Sheldon), a fungus of economic importance that causes Fusarium ear rot (FER) (Munkvold *et al*., 1997) and produces fumonisin mycotoxins in kernels (Gelderblom *et al*., 1988). FER reduces the quantity and quality of maize kernels by displaying scattered groups of mouldy kernels that may turn pink or salmon-coloured (White, 1999), whereas fumonisins are toxic metabolites harmful to human and animal health (Gelderblom *et al*., 1988; Marasas, 1995). Other *Fusarium* spp. such as *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas; *F. proliferatum* Matsushima, Nirenberg and *F. temperatum* Scauflaire & Munaut also occur in maize (Ncube *et al*., 2011; Janse van Rensburg, 2012; Schoeman, 2014) and cause FER (Leslie and Summerell, 2006; Zhang *et al*., 2014). Of these, *F. proliferatum* also produces fumonisins (Leslie and Summerell, 2006).

Maize production in South Africa is furthermore affected by stem borer infestations. *Busseola fusca* Fuller (Lepidoptera: Noctuidae) is the most injurious pest of maize in the country (Van Rensburg *et al*., 1988), and is endemic in commercial maize production areas (Kfir, 2000; 2002). *Busseola fusca* moths are known to oviposition on maize plants 4 weeks after plant emergence (Van Rensburg *et al*., 1987; Van Rensburg *et al*., 1989) by laying their eggs on unfurled leaves. The eggs then hatch into larvae during the early vegetative growth stages to feed on whorl tissue of young maize plants, before tunnelling into the maize stem where they cause severe damage (Kaufman, 1983). Tunnelling by stem borers can disrupt transport of water and translocation of nutrients in the xylem and phloem of the plant. It can also cause stem diseases due to pathogens gaining entrance into the plant through wounds produced during feeding (Sobek and Munkvold, 1999).

During feeding, insects produce waste products that contain both undigested feed from the gut as well as metabolic excretions such as urine from the Malpighian tubules (Gullan and Cranston, 2000). These waste products affect their interactions with other organisms, potential predators or prey (Weiss, 2006). Insects, therefore, possess a range of behavioural and morphological adaptations related to waste disposal which affect predator-prey interactions, hygiene, habitat location, reproduction, feeding and shelter construction (Weiss, 2006). Lepidopteran larvae, for instance, actively separate themselves from their frass by moving frass shorter distances when head-butting pellets. This behaviour could reduce exposure to pathogens or remove olfactory stimuli (cues) used by natural predators (Weiss, 2006). Larval

frass produced by *B. fusca* and *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae), for instance, have been shown to elicit a characteristic host-seeking response by *Cotesia flavipes* Cameron (Hymenoptera: Braconidae), an endoparasitoid used in the biological control of cereal stem borers (Van Leerdam *et al*., 1985; Potting *et al*., 1997).

Studies on the colonisation of maize stems with fungi have shown that fungal colonisation varied according to the location where the crop is produced. Fumonisin-producing *Fusarium* spp. were highly prevalent in Nigerian maize samples (Köhl *et al*., 2015), whereas *F. graminearum* Schwabe and *Fusarium avenaceum* Fries, Sacc. were most prevalent in samples from The Netherlands, and *F. verticillioides* and *F. proliferatum* Matsushima, Nirenberg in samples from Italy (Köhl *et al*., 2015). *Acremonium* sp. Link was found to be abundant in stems of maize which were poorly colonised by pathogenic *Fusarium* spp. in Italy (Köhl *et al*., 2015). Other fungi associated with maize stems include *Colletotrichum graminicola* (Ces.) G.W. Wils. (Gatch and Munkvold, 2002), *Stenocarpella maydis* Berk. (Flett *et al*., 1992; Gatch and Munkvold, 2002) and *Macrophomina phaseolina* (Tassi) Goid. (Kaiser and Das, 1988).

The fungal composition of *B. fusca* frass has not been investigated before. Some fungi in insect frass might benefit maize plants, while others could be deleterious and attract insects. The aim of this study, therefore, were to determine the mycoflora related to the frass of *B. fusca* larvae in maize stems.

MATERIALS AND METHODS

Collection of mycoflora in *B. fusca* larval frass

Maize plants with visible *B. fusca* damage were collected at the 6-8th leaf stages from maize fields at Kroonstad (27˚19'08.0"S; 27˚08'34.4"E) (11 plants, conservation tillage) in the Free State province, Sannieshof A (26˚45'09.7"S; 25˚48'53.6"E) (30 plants, conservation tillage), Sannieshof B (26˚45'09.7"S; 25˚48'53.6"E) (26 plants, conventional tillage) and Coligny (26˚46'51.5"S; 25˚53'16.4"E) (30 plants, conservation tillage) in the North West province. These localities are prone to high levels of natural fumonisin contamination (Janse van Rensburg, 2012), with *B. fusca* being endemic to these maize-producing regions (Kfir *et al*., 2002). Sterile surgical blades were used to open each stem, and all visible *B. fusca* frass (approximately 100 mg) was collected and placed into sterile Eppendorf tubes. One gram of *B. fusca* frass collected from each plant was then diluted with 9 ml of sterile distilled water, and a serial dilution prepared to 10^{-2}. The frass suspensions were thereafter plated out by transferring 1 ml of each suspension onto ½-strength potato dextrose agar (PDA) (Leslie and Summerell, 2006). After incubation for 2-3 days at 25˚C, the growing colonies were purified, single-spored and grouped according to growth morphology and colour.

Identification of mycoflora in *B. fusca* larval frass

Morphological species identification

Fungal isolates obtained from the survey were identified under a light Axioskop® Routine microscope (Carl Zeiss, Oberkochen, Germany) using morphological features common for *Aspergillus* spp. (Klich and Pitt, 1988), *Fusarium* spp. (Nelson *et al*., 1983; Leslie and Summerell, 2006) and other fungal species (Sutton, 1980).

Molecular identification

Fungal isolates retrieved from *B. fusca* frass were cultured on ½-strength PDA at 25˚C for 7 days in the dark, for DNA extraction. Mycelia was collected from each culture by scraping off the hyphae and spores with a sterile scalpel, whereafter it was deposited in sterile Eppendorf tubes. The mycelia was then ground with approximately 10 µg sterile, chemically-treated sand, followed by DNA isolation using the DNeasy plant mini-extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

Fungal DNA was amplified using primers for the translation elongation factor 1-α (*TEF1*) gene region for *Fusarium* spp. (EF-1 and EF-2) (O'Donnell *et al*., 1998), the RNA polymerase second largest subunit (*RPB2*) gene region for *Aspergillus* and *Talaromyces* spp. (RPB2-5F2 and fRPB2-7cR) (Liu *et al*., 1999; Sung *et al*., 2007), and the internal transfer spacer regions 1 and 2 and intervening 5.8S nrDNA (*ITS*) for *Acremonium* spp. and *Mucor* spp. (V9G and *ITS4*) (White *et al*., 1990; De Hoog and Van den Ende, 1998). The PCRs were performed using a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a total reaction volume of 25 µl. The PCR mixtures consisted of 2 µl genomic DNA, 10x Dream*Taq* reaction buffer with 2 mM $MgCl_2$ (Inqaba Biotech, Pretoria, South Africa), 125 µM of each dNTP, 0.4 µM of each primer, and 0.625 U Dream*Taq* polymerase (Inqaba Biotech). Conditions for PCR amplification of the *ITS* gene region consisted of an initial denaturation of 5 min at 94˚C; followed by 35 cycles of 30 s at 94˚C, 30 s at 48˚C and 60 s at 72˚C; and a final elongation step of 7 min at 72˚C. Amplification conditions for the *TEF1* gene region differed from the *ITS* region and included 40 cycles, with an annealing temperature of 59˚C, and an extension step of 45 s at 72˚C. The partial *RPB2* gene region was amplified with a touchdown PCR protocol of five cycles of 45 s at 94˚C, 45 s at 62˚C and 60 s at 72˚C, followed by five cycles with a 60˚C annealing temperature and 25 cycles with a 55˚C annealing temperature, and a final elongation step of 7 min at 72˚C.

PCR amplicons were purified using a QIAquick PCR Purification kit (Qiagen, Hilden, Germany), whereafter they were sequenced at Inqaba Biotech in a single direction using the PCR primers and the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). The sequences were then analysed with an ABI Prism 3730XL Sequencer (Applied Biosystems). Consensus sequences were created from the forward sequences using

BioEdit v7.0.9.0 (www.mbio.ncsu.edu/BioEdit/BioEdit.html). They were then submitted to the basic local alignment search tool (BLAST) on GenBank at National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/), MycoBank (http://www.mycobank.org/), and the Fusarium MLST database (http://www.cbs.knaw.nl/fusarium/) in order to confirm the identity of the isolates.

Phylogenetic analyses

Reference sequences for *Fusarium* spp. (19 reference sequences) and *Aspergillus* spp. (12 reference sequences) were selected on the basis of BLAST results. Multiple sequence alignments were generated with MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/index.html). Phylogenetic analyses was based on parsimony using PAUP 4.0 (Phylogenetic analysis using parsimony and other methods version 4; Swofford, 2002). Heuristic searches were performed with random addition of sequences (1 000 replicates), tree bisection-reconnection (TBR) branch swapping, and MULPAR effective and MaxTrees set to auto-increase. The consistency (CI) and retention (RI) indices were determined for the data sets. The phylogenetic trees for *Fusarium* spp. were rooted with *Cylindrocarpon candidum* Link Wollenw. (syn = *Fusarium candidum* Link Sacc.) and *Cylindrocarpon cylindroides* Wollenw. (syn = *Fusarium cylindroides* Link Sacc.), based on the work of Lombard *et al*. (2015). The trees for *Aspergillus* spp. were rooted with *Aspergillus paradoxus* Fennell & Raper. Bootstrap analyses were performed to determine branching point confidence intervals (1 000 replicates) for the most parsimonious trees generated for the data sets.

Dissemination of *F. verticillioides* spores in *B. fusca* larval frass

The possible spread of *F. verticillioides* spores in *B. fusca* frass from the whorl into the stem was investigated in the greenhouse and field. Two treatments were applied: (i) inoculation with *F. verticillioides* and infestation with *B. fusca*, and (ii) infestation with *B. fusca* alone (control treatment), with 30 maize plants for each treatment. The greenhouse trial was conducted in steam-sterilised potting soil at the greenhouse facilities of the North-West University in Potchefstroom, whereas the field trial was planted at the ARC-GCI experimental farm in Potchefstroom during the 2012/13 season. Endophytic *Fusarium* spp. were eradicated before planting by placing the seeds in sterile distilled water for 4 hrs at room temperature, then in water at 55°C for 5 min, before they were cooled down in water at room temperature for a few seconds (Daniels, 1983). The seeds were then air-drying overnight (Daniels, 1983).

To determine the presence of fungal spores in *B. fusca* larval frass, *F. verticillioides* isolate MRC826 was produced in Armstrong *Fusarium* medium (Booth, 1971) to a final concentration of 2×10^6 spores ml^{-1}. The *F. verticillioides* suspension (approximately 4 ml) was then sprayed into the whorl of maize plants 4 weeks after plant emergence. Two days later, aliquots of 5-10

neonate *B. fusca* larvae were deposited into the whorl using a mechanical applicator that was calibrated to release the stated number of neonate larvae. These larvae were mass produced earlier at the rearing facility of the ARC-GCI in Potchefstroom (Van Rensburg and Van Rensburg, 1993). Each stem was opened 3 weeks after *B. fusca* infestation using sterile surgical blades, and the frass was collected from feeding channels and placed into an autoclaved Eppendorf tube.

Target DNA of fumonisin-producing *Fusarium* spp. present in the frass was quantified using quantitative real-time PCR (qPCR). Fungal DNA was extracted from approximately 50-100 mg pulverized frass with a cetyl trimethyl-ammonium bromide (CTAB; Sigma-Aldrich, St Louis, Missouri) protocol (Doyle and Doyle, 1987). The qPCR reaction was performed with the *FUM1*-probe and Taqfum-2F and Vpgen-3R primers (Waalwijk *et al*., 2008), as described in Chapter 2.

Statistical analysis

Mean (ng fungal DNA / 0.5 mg milled maize sample) DNA of fumonisin-producing *Fusarium* spp. were subjected to a Two-sample *t*-test using GenStat 15th edition (VSN, International, Hemel Hempstead, UK).

RESULTS

Fungal species isolated from *B. fusca* frass

A total of 70 fungal isolates were retrieved from *B. fusca* frass. These included *Acremonium zeae* Gams & Sumner; *Aspergillus flavus* Link ex Fr; *A. niger* Tiegh; *F. chlamydosporum* Wollenw. & Reinking; *F. incarnatum-equiseti* species complex (FIESC) O'Donnell *et al.*; *F. oxysporum* Schlecht.; *F. subglutinans*; *F. verticillioides*; members of the *Gibberella fujikuroi* species complex (GFSC) Sawada; *Mucor circinelloides* Tiegh.; *Rhizopus oryzae* Went & Prins.; and *Talaromyces flavus* Klöcker (Table 1). More than one fungal species were sometimes associated with a single frass sample. The *Fusarium* spp. most frequently isolated were *F. oxysporum* (20 isolates), *F. subglutinans* (seven isolates) and *F. verticillioides* (five isolates). *Fusarium oxysporum* and *Ac. zeae* were isolated from *B. fusca* frass in all localities sampled, whereas *F. verticillioides* and *A. flavus* were isolated in two of the four localities, namely Coligny and Sannieshof A (Fig. 1). *Fusarium oxysporum* and the *Gibberella fujikuroi* species complex were the only *Fusarium* spp. isolated from *B. fusca* frass in Sannieshof B whereas in the neighbouring Sannieshof A, *F. chlamydosporum*, *F. oxysporum*, *F. subglutinans* and *F. verticillioides* were isolated (Fig. 1). *Fusarium subglutinans* (Kroonstad, Coligny and Sannieshof A), *F. chlamydosporum* (Sannieshof A) and *Fusarium incarnatum-equiseti* complex (Coligny) were also isolated from *B. fusca* frass (Fig. 1). All isolates were

deposited in the National Collection of Fungi (NCF), Agricultural Research Council-Plant Protection Research Institute, Roodeplaat, Pretoria.

Phylogenetic analysis showed that most *Fusarium* spp. grouped into single distinct clades with high bootstrap values. The *F. oxysporum* isolates were grouped into five lineages with high bootstrap support (Fig. 2). *Fusarium subglutinans* also grouped into two lineages. The *RPB2* gene sequencing divided the *Aspergillus* isolates (Fig. 3) into two species, *A. niger* and *A. flavus*. Isolates of *A. niger* were divided into two lineages, and isolates of *A. flavus* all grouped into a single lineage. *Acremonium zeae*, *Mucor circinelloides*, *Rhizopus oryzae*, and *Talaromyces flavus* did not divide into separate lineages.

The presence of *F. verticillioides* spores in *B. fusca* larval frass and its possible disseminating within the maize plant in maize

In the greenhouse trial, frass collected from plants inoculated with *F. verticillioides* had significantly more target DNA of fumonisin-producing *Fusarium* spp. (*P*-value = 0.007; degrees of freedom = 20) than in the frass of larvae in the uninoculated control (Fig. 4). However, results from the field trial showed that there was no significant difference (*P*-value = 0.21; degrees of freedom = 35) between the quantity of target DNA of fumonisin-producing *Fusarium* spp. in the frass collected from within the plants that were inoculated with *F. verticillioides* and those that were uninoculated.

DISCUSSION

Several fungal species isolated from *B. fusca* frass are commonly associated with maize plants; some as endophytes and others as pathogens. These include *F. verticillioides* (Munkvold and Carlton, 1997; Yates *et al*., 1997), *F. oxysporum* (Leslie and Summerell, 2006) and *Ac. zeae* (Wicklow *et al*., 2005); all common endophytes of maize plants. *Fusarium verticillioides* is an endophyte that can transition from symptomless infection to necrotrophic pathogenicity in wounded maize stems and kernels (Rutherford *et al*., 2002). *Fusarium verticillioides* also infects maize ears through the silk channel which is the most important pathway (Munkvold and Carlton, 1997; Galperin *et al*., 2003), lateral roots (Oren *et al*., 2003) and biotic and abiotic wounds on maize ears (Munkvold *et al*., 1999; Robertson *et al*., 2011; Chapter 2). Endophytic isolates of *F. verticillioides* have been shown to suppress the growth of the maize smut pathogen, *Ustilago maydis* DC (Corda), by interfering with the early infection process and limiting disease development (Lee *et al*., 2009).

Fusarium oxysporum, the fungus most commonly associated with *B. fusca* frass in this study, often comprise non-pathogenic strains that can be used as biocontrol agents. Non-pathogenic *F. oxysporum* strains induced systemic resistance in banana (*Musa acuminata*

Colla) (Nel *et al*., 2006; Kidane, 2008; Belgrove *et al*., 2011) and tomato (*Solanum lycopersicum* L.) (Shishido *et al*., 2005) plants against pathogenic *F. oxysporum* strains by enhancing the plant's ability to defend itself against pathogen attack through the activation of the jasmonic acid, salicylic acid and ethylene pathways (Sticher *et al*., 1997; Benhamou and Garand, 2001). Non-pathogenic *F. oxysporum* also produces β-caryophyllene that attract natural predators of stem borers (Köllner *et al*., 2008), while others suppress nematodes that can cause wounds for root pathogens to infect (Mwaura *et al*., 2010; Waweru, *et al*., 2014).

Pathogenic fungi, such as *A. flavus* (Campbell and White, 1995; Ehrlich *et al*., 2010) and *F. subglutinans* (Vigier *et al*., 2009) were also isolated from the frass. *Fusarium subglutinans* causes FER and *A. flavus* cause Aspergillus ear rot in maize (Campbell and White, 1995; Ehrlich *et al*., 2010). *Aspergillus flavus* infect maize ears through the silk channel (Widstrom, 1979) and wounds produced by stem borers (Williams *et al*., 2002). Moreover, *F. oxysporum* strains have been shown to produce the beauvericin mycotoxin (Moretti *et al*., 2002; Zhan *et al*., 2007). Beauvericin has been shown to be toxic to *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) (Cherry *et al*., 2004) and *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) stem borers (Bing and Lewis, 1991; 1992) in maize. Therefore the beauvericin-producing *F. oxysporum* strains could potentially be utilised in the biological control of *B. fusca* larvae.

Acremonium zeae was isolated from the *B. fusca* frass in all localities when compared to *F. verticillioides*. *Acremonium zeae* is known to be antagonistic to *F. verticillioides* through the production of pyrrocidines A and B antibiotics (Wicklow *et al*., 2005), and has been associated with reduced colonisation of maize stem with the fumonisin-producing *F. verticillioides* and *F. proliferatum* (Köhl *et al*., 2015). Therefore, *Ac. zeae* could potentially be utilised as a biological control agent in the reduction of stem and ear rot caused by *F. verticillioides*, *F. subglutinans*, *F. temperatum* and other members of the GFSC (Varela *et al*., 2013), as well as the reduction of their associated mycotoxin production. The occurrence of *Ac. zeae* in *B. fusca* frass suggests that suppression of *F. verticillioides* with *Ac. zeae* should be further investigated *in planta*, as the latter can serve as an important biocontrol agent in subsistence farming systems where farmers plant farm-saved seed that are infected with *F. verticillioides* (Ncube, 2008). Moreover, *Ac. zeae* also interferes with *A. flavus* infection and aflatoxin contamination of pre-harvest maize kernels (Wicklow *et al*., 2005). *Aspergillus flavus* is not considered an important ear rot pathogen of maize in commercial maize farming in South Africa, but is of importance in subsistence farming systems (Ncube, 2008).

Aspergillus niger was an interesting fungus associated with *B. fusca* frass in this study, as it has recently been found to produce fumonisins (Frisvad *et al*., 2007), particularly fumonisin B_2, in maize kernels (Logrieco *et al*., 2014). Its potential role in the contamination of maize kernels with fumonisin in South Africa, therefore, needs to be further investigated.

Moreover, determining the cause of fumonisin production by either *F. verticillioides* and/or *A. niger* can be important in mitigating the risks of fumonisin contamination in maize kernels since most studies on fumonisin production in maize are focused on *F. verticillioides* as the causative organism in South Africa.

Differences in *Fusarium* spp. composition in the *B. fusca* frass between the two localities in Sannieshof could possibly be due to differences in farming practices. Conservation tillage was practiced in Sannieshof A, while conventional tillage was followed in Sannieshof B. A higher diversity of *Fusarium* spp. has been found in conservation tillage systems than conventional tillage systems (Steinkellner and Langer, 2004). Moreover, FER pathogens: *F. verticillioides*, *F. proliferatum* and *F. subglutinans* survive longer in fields with surface maize residue when compared to fields with buried residues (Cotten and Munkvold, 1998).

The significantly high levels of target DNA of fumonisin-producing *Fusarium* spp. in the frass collected from the *F. verticillioides*-inoculated plants compared to uninoculated plants in the greenhouse indicate that *B. fusca* larvae possibly disseminated *F. verticillioides* spores from the whorls into the stems of maize plants. There were no significant differences in the quantity of target DNA of fumonisin-producing *Fusarium* spp. between *F. verticillioides*-inoculated plants and uninoculated plants in the field trial, possibly due to natural *F. verticillioides* infection through rain splash and air movement onto the lower nodes of the stem (Munkvold and Desjardins, 1997). Co-infection with *A. niger*, which also contains gene sequences analogous to the fumonisin biosynthesis genes found in *F. verticillioides* and *F. proliferatum* (Baker, 2006; Pel *et al*., 2007; Susca *et al*., 2016).

This study was the first to investigate fungal pathogens in *B. fusca* frass. The results indicated that *B. fusca* frass was a reservoir of different fungal species; some with antagonistic properties against maize pathogens, and others pathogenic to maize. It further showed that *B. fusca* infestation of maize stems was associated with higher levels of fumonisin-producing *Fusarium* spp. in larval frass when *F. verticillioides* was present on the plant. Therefore *B. fusca* infestation potentially has an ecological role in modulating the dissemination of maize fungal pathogens through its frass. Moreover, the presence of fungal endophytes in the frass may reflect the mycoflora inherently present in the plant and their growth is possibly promoted in the frass.

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Table 1. Fungi isolated from the *Busseola fusca* frass that were identified based on partial sequenced *ITS*, *TEF1* and *RPB2* gene regions

Name	Gene region used	Number of isolates
Acremonium zeae	*ITS*	14
Aspergillus flavus	*RPB2*	8
Aspergillus niger	*RPB2*	4
Fusarium chlamydosporum	*TEF*1	1
Fusarium incarnatum-equiseti species complex (FIESC)	*TEF*1	1
Fusarium oxysporum	*TEF*1	20
Fusarium subglutinans	*TEF*1	7
Fusarium verticillioides	*TEF*1	5
Gibberella fujikuroi species complex (GFSC)	*TEF1*	1
Mucor circinelloides	*ITS*	2
Rhizopus oryzae	*ITS*	1
Talaromyces flavus	*RPB2*	6

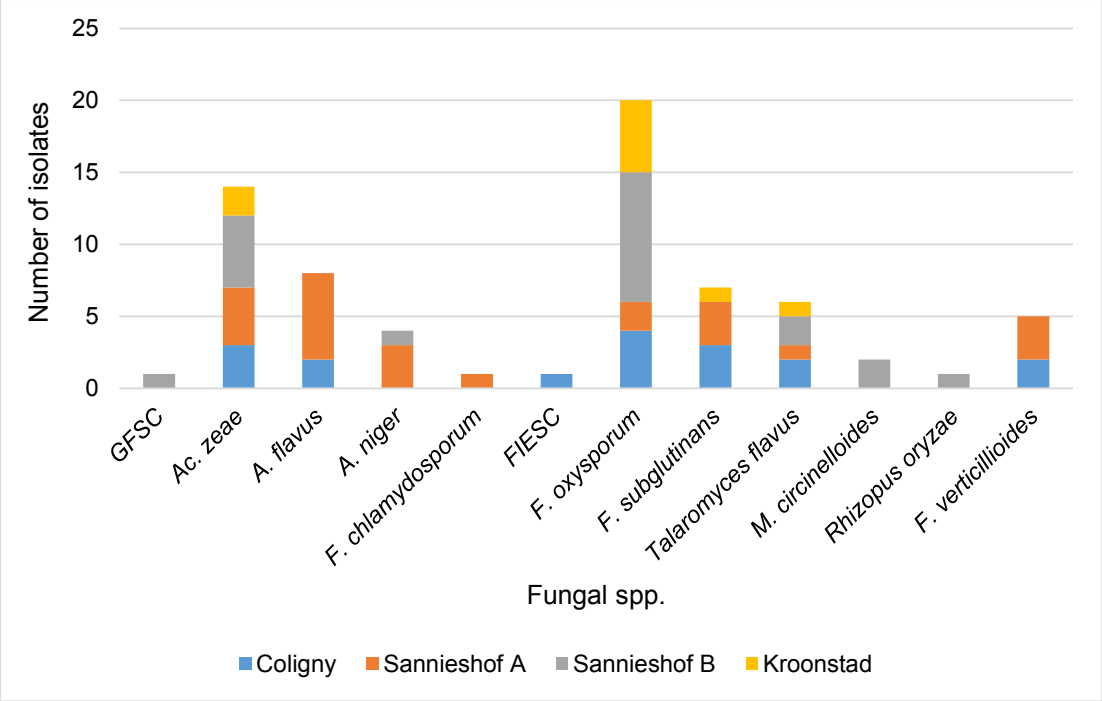

Figure 1. Fungal species isolated from *Busseola fusca* frass in three maize-growing districts of South Africa.

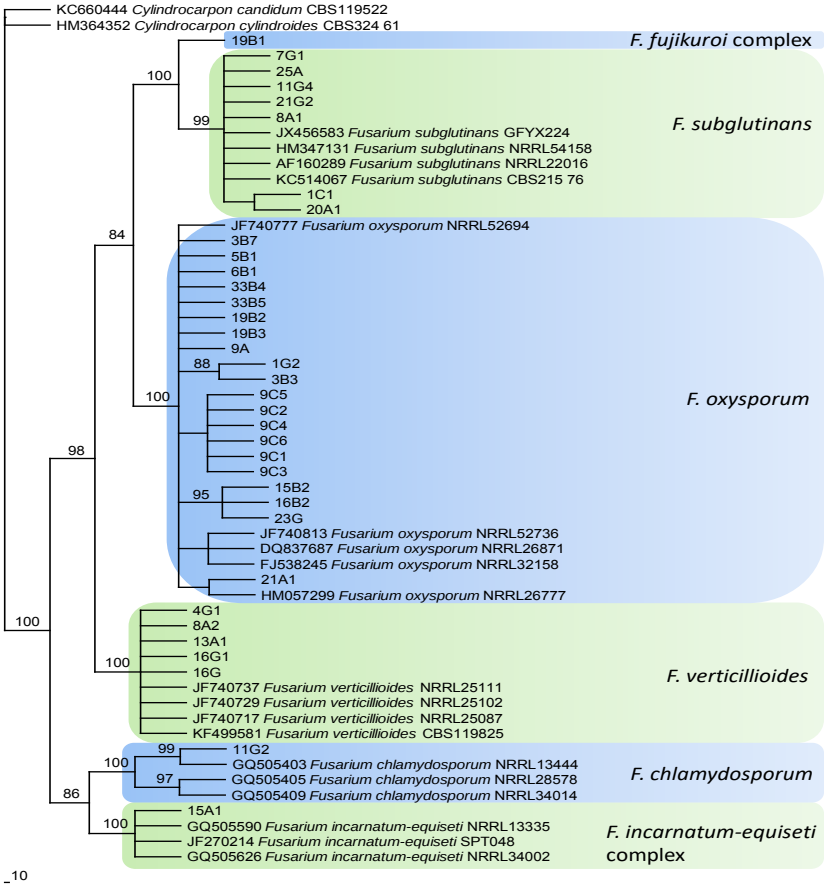

Figure 2. Phylogenetic tree for *Fusarium* spp. isolated from *Busseola fusca* frass, constructed using the translation elongation factor-1 (*TEF-1*) gene region sequences (Consistency index [CI] = 0.7939; Retention index [RI] = 0.9500).

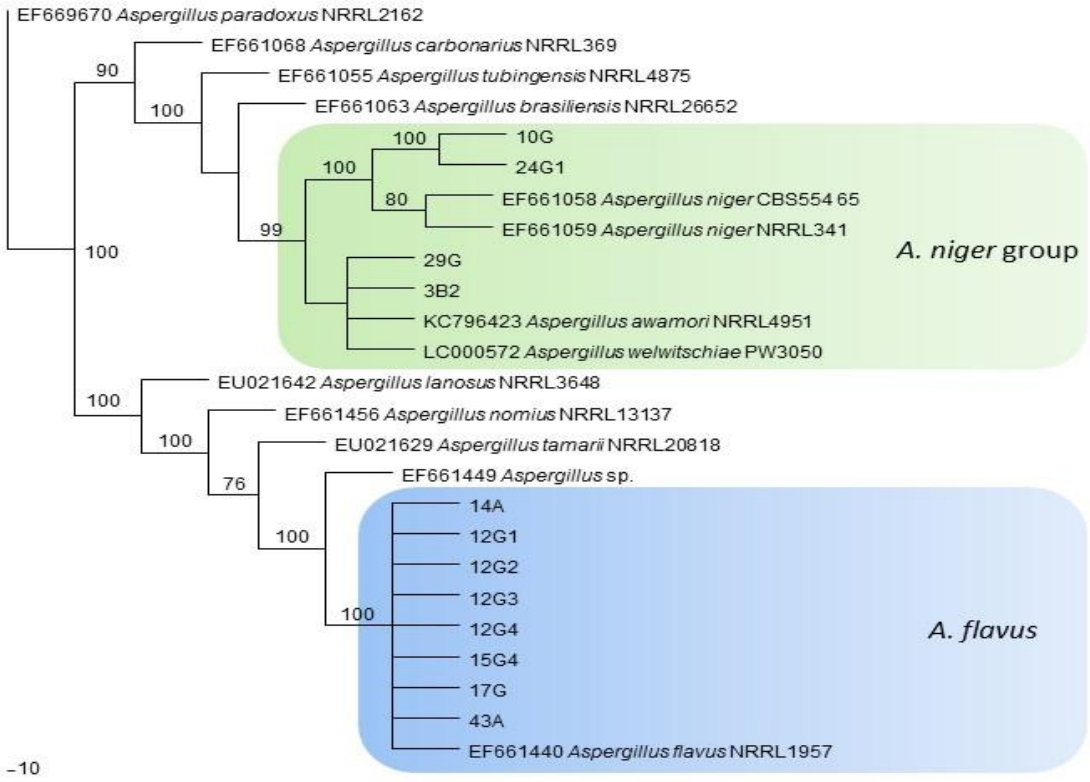

Figure 3. Phylogenetic tree for *Aspergillus* spp. isolated from *Busseola fusca* frass, constructed using the RNA polymerase second largest subunit (*RPB2*) gene region sequences (Consistency index [CI] = 0.6502; Retention index [RI] = 0.8987).

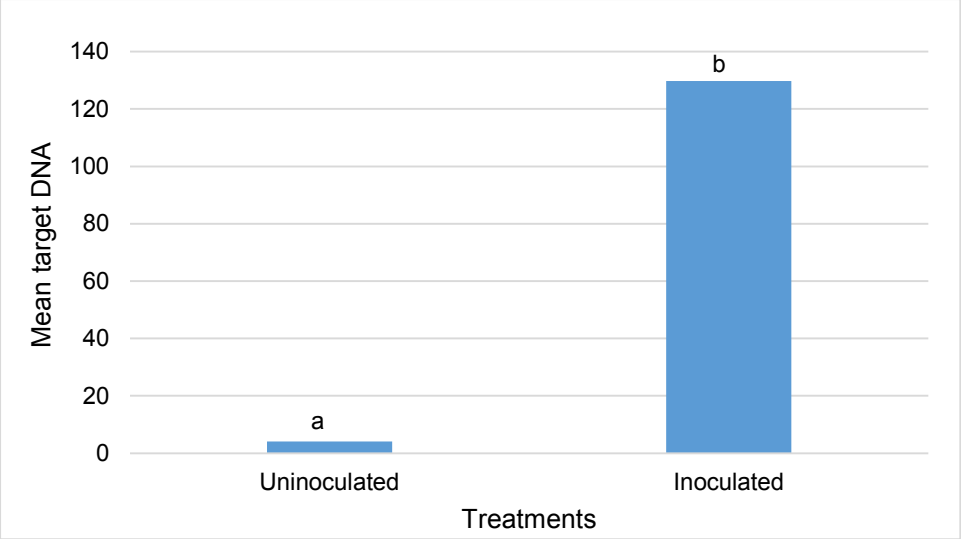

Figure 4. Mean target DNA (pg fungal DNA / 0.5 mg frass sample) of fumonisin-producing *Fusarium* spp. in the frass of the control treatment (uninoculated) and the treatment where maize plants were inoculated with *F. verticillioides* MRC826 (inoculated) and infested with *Busseola fusca* larvae in the greenhouse trial. The letters represent the significant difference between the uninoculated and inoculated treatments (*P*-value = 0.007; degrees of freedom = 20) using the Two-sample *t*-test.

CHAPTER 5

Conclusion

Approximately 9 million tons of maize is consumed as food and feed in South Africa annually (DAFF, 2014). To meet the future demand for food and feed, however, innovative ways of producing agricultural crops will have to be developed. These innovations include a better understanding of plant, insect and pathogen interactions for disease management. This study has investigated the interaction between the maize plants, the fungus *Fusarium verticillioides* Sacc. Nirenberg (syn = *F. moniliforme* Sheldon) and the stem borer *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) in an attempt to reduce Fusarium ear rot (FER) and fumonisin contamination of kernels by understanding the effect of wounding, genetic modification, pesticide application and vectoring of plant pathogens by insect larvae.

From this study it is apparent that *B. fusca* infestation was not the predominant modulating factor for the occurrence of FER and fumonisin production. *Busseola fusca* created wounds that enabled infection of maize plants with *F. verticillioides*, but its sporadic feeding on maize ears limited its association with disease and fumonisin production. FER development and fumonisin production by *F. verticillioides* also varied between seasons, thereby demonstrating the predominant modulating effect of environmental conditions. The cultivation of genetically modified maize (*Bt* maize) that is toxic to *B*. *fusca* larvae reduced the incidence of FER and fumonisin production in kernels under natural field conditions. However, *Bt* maize did not affect the colonisation of maize ears by fumonisin-producing *Fusarium* spp. and fumonisin production in *F. verticillioides*-inoculated maize ears. This indicated that the effect of *Bt* maize on fumonisin production in maize ears is indirectly associated with its control of stem borer damage.

The recent discovery of *B*. *fusca* resistance to *Bt* maize (*MON810*) with a single Cry1Ab crystal protein suggests that this technology should not be the only one available for the management of *B. fusca*. The toxicity of the *Bt* toxin could be augmented by introducing the defensive Mir1-CP protein (Pechan *et al*., 2000) from Antiguan maize germplasm into susceptible maize hybrids (Hilbeck and Otto, 2015). The pyramid *Bt* maize (*MON89034*), which expresses two different crystal proteins Cry1A.105 and Cry2Ab, was commercially released in South Africa during 2011 (Van den Berg, 2012). This is important not only for the control of stem borers, but also for the indirect control of FER and fumonisin production. It remains to be seen whether *B. fusca* will develop resistance to the newly released pyramided genes in the country.

The application of Benfuracarb to control *B. fusca* reduced the incidence of FER and production of fumonisin in maize kernels, but Beta-cyfluthrin had some limitations. For instance, *B. fusca* larvae primarily feed in the whorls of plants until the fourth instars, therefore the application of Beta-cyfluthrin in the whorl is effective only until shortly before tasselling because active ingredients cannot reach inside the stem where the stem borers reside (Van Rensburg *et al*., 1987). Benfuracarb can be considered as an insecticide for use in an integrated disease management strategy since it reduced *B*. *fusca* damage, and therewith FER and fumonisins in maize. Benfuracarb also has a dual role as it can control nematodes (McDonald *et al*., 1987) in addition to *B. fusca* (Van Rensburg *et al.*, 1991). The pesticide is effective over the entire pre-tasselling period (Van Rensburg *et al*., 1991) and reaches stem borers in all plant tissues when applied as a seed dressing. It is, however, toxic if inhaled or swallowed, and is also very toxic to aquatic organisms (EC, 2008), thereby rendering its application above-ground dangerous. The application of the recently released fluopyram, a new and unique pyridinyl ethyl benzamid nematicide (Broeksma *et al*., 2014) and broad-spectrum fungicide (Sierotzki and Scalliet, 2013), could potentially result in the control of pathogens such as *F. verticillioides* and nematodes. This may reduce nematode wounding that might promote *F. verticillioides* infection through wounded lateral roots.

Species of *Acremonium*, *Aspergillus*, *Fusarium*, *Mucor*, *Rhizopus* and *Talaromyces* were associated with *B. fusca* frass, indicating an important reservoir of potentially beneficial and detrimental organisms associated with the insect. The original contact between the fungi and *B. fusca* was not investigated, although the study suggested that *F. verticillioides* had been vectored from the whorls into the stem. *Fusarium verticillioides* was not the only ear rot and mycotoxin-producing fungus associated with frass. *Aspergillus niger* (Tiegh), another fumonisin-producing fungus (Frisvad *et al*., 2007), was also found in the frass. The fungi found in the frass of *B*. *fusca* may either have entered the maize stem through wounds, as systemic infections, or being vectored by insect larvae while feeding on other plant tissues. The presence of *A. niger* in *B. fusca* frass suggests that it is important to determine the origin of fumonisin contamination in maize (either from *F. verticillioides* or *A. niger* or both) in South Africa, since *A. niger* has also been shown to produce fumonisins. The presence of the maize protective endophyte *Ac. zeae* in frass has potential applications in the biological control of *F. verticillioides*, as it produces pyrrocidines A and B antibiotics which are antagonistic to *F. verticillioides*.

Mycotoxins and mycotoxin-producing fungi have a potential in the development of biopesticides. Mycotoxigenic fungi, such as *F. sacchari* Gams, are detrimental to the survival of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) in maize (McFarlane *et al*., 2009) while fusaproliferin that is produced by *F. subglutinans* (Fumero *et al*., 2015) is toxic to *Artemia salina* L. and SF-9 *Spodoptera frugiperda* (Lepidoptera: Noctuidae) cell lines (Logrieco *et al*.,

1996). The beauvericin mycotoxin that is produced by endophytic *Beauveria bassiana* (Balsamo) Vuillemin; *Fusarium concentricum* Nirenberg & O'Donnell; *F. guttiforme* Nirenberg & O'Donnell; *F. circinatum* Nirenberg & O'Donnell (Fotso *et al*., 2002); and *F. temperatum* Scauflaire & Munaut (Scauflaire *et al*., 2011; 2012), has been shown to be toxic to *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) (Cherry *et al*., 2004) and *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) stem borers (Bing and Lewis, 1991; 1992) in maize. *Beauveria bassiana* metabolites were also found to be toxic to neonate *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) larvae (Leckie *et al*., 2008) and *H. armigera* Hübner (Ritu *et al*., 2012). Nevertheless, the application of beauvericin as a biopesticides is however limited due to its phytotoxicity (Paciolla *et al*., 2004; Šrobárová *et al*., 2009; Pavlovkin *et al*., 2012).

Many fungal species rely on insects for spore dispersal and produce volatile organic compounds (VOCs) mimicking those produced by flowers to attract insect pollinators (Bruce *et al*., 2005). Fungal endophytes may have either a positive or a negative effects on herbivores, and could be an important factor causing unpredictable herbivore feeding (Saikkonen *et al*., 2013). Sétamou *et al*. (1998) reported a significant interaction between infestation of maize with *Mussidia nigrivenella* (Ragonot) (Lepidoptera: Pyralidae) and *Aspergillus flavus* Link ex Fr infection. Inoculation with *A. flavus* resulted in higher levels of aflatoxin B_1 production when *A. flavus* was associated with *M. nigrivenella* borers than with the fungus alone in pre-harvest maize in Benin. Schulthess *et al*. (2002) found a significant positive correlation between ear/stem *F. verticillioides* infection and *E. saccharina* in maize in Benin also.

It has been reported that *E. saccharina* larvae show a marked preference for certain *Fusarium* isolates while avoiding others in olfactory choice assays of maize kernels inoculated with a range of *Fusarium* isolates (McFarlane *et al*., 2009). Fungal secondary metabolites also shape food choice behaviour thereby affecting population dynamics of fungivores such as the springtail, *Folsomia candida* Lubbock (Insecta, Collembola) (Rohlfs *et al*., 2007). The loss of secondary metabolite *LaeA* global regulator in *Aspergillus nidulans* (Eidam) G. Winter increased its preference for *F. candida* predation (Rohlfs *et al*., 2007). Moreover, the consumption of the mutant yielded a reproductive advantage to the arthropod but was detrimental to fungal biomass compared with a wild-type fungus capable of producing secondary metabolites (Rohlfs *et al*., 2007). *Epichloë funkii* (K.D. Craven & Schardl.) J.F. White, an endophyte of sleepygrass [*Achnatherum robustum* (Vasey) Barkworth] produces alkaloid compounds that are toxic to invertebrate or vertebrate herbivores (Shymanovich *et al*., 2015). The presence of *E. funkii* can affect herbivore abundances and species richness due to toxic alkaloids produced (Jani *et al*., 2010).

Microorganisms and/or their secondary metabolites occurring within the frass could be

used as biocontrol agents in the control of *B. fusca*. Moreover, other secondary metabolites such as beauvericin are toxic to stem borers as well as plants, thus reducing their potential as biological control agents for stem borers. However, screening of *F. oxysporum* isolates occurring in *B. fusca* frass for beauvericin production could determine the extent of beauvericin exposure in maize plants.

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