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

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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. The African stem borer (*Busseola fusca*) is a pest of economic importance in 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 and fumonisin production in a *Bt* hybrid (MON810 event) and its *B. fusca*-susceptible non-*Bt* isohybrid. Field trials were conducted over three seasons using a randomised complete block design with six replicates per treatment. The effect of Beta-cyfluthrin (non-systemic, granular) and Benfuracarb (systemic, seed treatment) insecticide applications on the incidence of Fusarium ear rot and fumonisin production in maize was also determined in an unrelated 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. Maize ears were harvested at physiological maturity and Fusarium ear rot, total fumonisin levels, stem borer damage and target DNA of fumonisin-producing *Fusarium* spp. quantified. Significantly less Fusarium ear rot 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. extracted from maize plants. Benfuracarb application to control stem borer infestation resulted in a significant reduction in Fusarium ear rot 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 indicated that *Bt* maize and the application of Benfuracarb reduce *B. fusca* damage to maize ears thereby indirectly reducing Fusarium ear rot and fumonisin production. However, this was not consistent over seasons due to differences in climatic conditions.

1. 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

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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 losses of between 10 and 60% (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 2000 m above sea level (Abate et al., 2000). *Busseola fusca* larvae feed mainly in the whorls of plants 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). *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 stem borers of lesser importance in South Africa are *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) (Kfir et al., 2002) and *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) (Van den Berg, 1997; Van den Berg and Drinkwater, 2000), which cause similar damage as *B. fusca*.

Damage caused by *B. fusca* larvae can be reduced through the application of insecticides (Beyene et al., 2011), crop residue management (Kfir et al., 2002), push-pull habitat manipulation and trap cropping (Khan et al., 2008; Van Den Berg and Van Hamburg, 2015) and biological control (Kfir et al., 2002). However, *B. fusca* damage is most effectively controlled by planting maize genetically modified maize with *Bacillus thuringiensis* (*Bt*) genes that encode for δ-endotoxin crystal proteins that are toxic to lepidopteran insects (Hellmich et al., 2008). *Bt* maize hybrids have been found to be less prone to Fusarium ear rot and fumonisin contamination than non-*Bt* maize hybrids after infestation with *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) 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; Bowers et al., 2014). *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). High levels of gene expression for the lipoxygenase-derived oxylipin genes protect plants against oxidative stress resulting in maize ears that are resistant to *F. verticillioides* colonisation (Maschietto et al., 2015).

Bt maize (MON810 event, expressing a single Cry1Ab gene) has been widely cultivated in South Africa to reduce *B. fusca* damage. Resistance in *B. fusca* larvae to the MON810 event has, however, occurred due to selection pressure derived from continuous exposure of larvae 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 event) 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 stacked-gene *Bt* hybrids containing MON89034 event which expresses the Cry1A.105 and Cry2Ab2 genes (Van den Berg et al., 2013). These stacked-gene hybrids that produce two different insecticidal proteins are a much more effective insect resistance management (IRM) tool (Van den Berg et al., 2013). Seed mixtures with different ratios of non-*Bt* and *Bt* maize seed have also been suggested as an IRM tool but may not be effective to delay resistance evolution in *B. fusca* (Erasmus et al., 2016).

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, successfully controls *B. fusca* and *C. partellus* larvae (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 (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 appearance results in poor control of *B. fusca* because insecticides are ineffective against larvae in stems (Van Rensburg et al., 1987b). The cryptic feeding behaviour of stem borer species inside plant stems adversely affects the efficacy of whorl applied insecticides (Slabbert and Van den Berg, 2009), making the use of *Bt* maize a viable option for pest management.

Busseola fusca damage has been reported to increase the incidence of Fusarium ear rot 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). This study, therefore, was conducted to elucidate the effect of *Bt* maize and insecticide application, as *B. fusca* control strategies, on Fusarium ear rot and fumonisin production in maize in South Africa.

2. Materials and methods

2.1. Experimental design

Field trials to evaluate the effect of *Bt* maize and insecticides on Fusarium ear rot 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. Field trials comprising of a commercial *Bt* maize hybrid (PAN6236B) expressing the MON810 event, and its insect-susceptible non-*Bt* isohybrid (PAN6126), were 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 event was the only commercially available *Bt* event for the control of stem borers in maize in South Africa at the time of the study.

Field trials in which insecticide efficacy was evaluated were also planted. These trials involved a non-*Bt* (conventional) maize hybrid (PAN6723), and was planted at the ARC-GCI experimental farm during 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. All trials were planted under conventional dry land conditions. Soil analysis was performed before planting to calculate the quantity of fertilisers required. Pre-

emergence (S-metolachlor) and post-emergence (thiadiazine) herbicides were applied for weed control according to the manufacturer's instructions.

The field trial of the *Bt* maize hybrid and its insect-susceptible non-*Bt* isohybrid had the following treatments: *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.* Benfuracarb, a systemic insecticide, was applied to maize seed prior to planting according to the manufacturer's instructions, while Beta-cyfluthrin, a non-systemic insecticide, was applied directly into the whorl according to the manufacturer's instructions. This was done on a weekly basis, beginning 2 weeks after seedling 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.

2.2. 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 (Lasec, Johannesburg, South Africa). The suspension was then spun at 1000 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 *B. fusca* larvae were produced at the mass rearing facility at the ARC-GCI in Potchefstroom according to the methods developed by 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 and 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 directly to stems, as is the case with early borer infestations (J.B.J. van Rensburg, personal communication).

2.3. Evaluation of Fusarium ear rot and B. fusca damage

Fusarium ear rot symptoms on each ear were visually rated as described by Enerson and Hunter (1980). Fusarium ear rot 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 on each ear was expressed as a percentage of the total ear surface. Fusarium ear rot ratings excluded tunnels that did not show visible ear rot symptoms. However, damaged kernels in tunnels with visible Fusarium ear rot symptoms were included in the ear rot ratings. Each ear from each experimental row (replicate) was rated individually, after which an average Fusarium ear rot score for each experimental row (replicate) was calculated. *Busseola fusca* damage was determined by measuring the cumulative feeding tunnel length (cm) on each ear upon harvest. Each ear was first rated individually, and the mean *B. fusca* damage was thereafter calculated for each experimental row.

2.4. Quantification of fumonisin-producing Fusarium spp.

The quantity of target DNA of fumonisin-producing *Fusarium* spp. was determined in a 0.5-mg sample of all milled maize kernels from each replicate using quantitative real-time PCR (qPCR) (Waalwijk et al., 2008). 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 to 99.9%, while the R^2 ranged from 0.992 to 0.997.

2.5. Fumonisin analysis

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.

2.6. 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 from January to April is important in the Fusarium ear rot and fumonisin context because it largely covers the period between silking and the dent stage at which Fusarium ear rot 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.

2.7. Statistical analyses

An analysis of variance (ANOVA) was performed on the data for Fusarium ear rot, fumonisin production, quantity of target DNA of fumonisin-producing *Fusarium* spp. and the cumulative tunnel length caused by *B. fusca* larvae (*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 Fusarium ear rot, and the quantity of target DNA of fumonisin-producing *Fusarium* spp., as well as between Fusarium ear rot and the quantity of target DNA of fumonisin-producing *Fusarium* spp. were performed using Statgraphics® 5 Plus (Statpoint Technologies Incorporated, Warrenton, VA, US) on a *Bt* hybrid (PAN6236B) across three seasons.

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

3. Results

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

3.1.1. Fusarium ear rot and B. fusca damage

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

3.1.2. Quantity 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 during the 2010/11 season.

3.1.3. 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*, irrespective of *B. fusca* infestation in the 2009/10 and 2010/11 seasons, but not in the 2011/12 season. There were, however, no significant differences in the quantity of fumonisins produced in maize kernels inoculated with *F. verticillioides* 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 was not observed 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

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

Hybrid	Season		
	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* and its non-*Bt* isohybrid following inoculation with *Fusarium verticillioides* and/or *Busseola fusca* compared to a non-inoculated control.

Treatment	Hybrid	
	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) caused by *Busseola fusca* in ears of a *Bt* and its non-*Bt* maize isohybrid.

Cultivar	Treatment	Season		
		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) target DNA of fumonisin-producing *Fusarium* spp. in both a *Bt* hybrid and its non-*Bt* isohybrid, comparing inoculation treatments and control across three seasons.

Treatment	Season		
	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* and non-*Bt* isohybrid.

Treatment	Season		
	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.

F. verticillioides with and without *B. fusca* infestation were significantly more contaminated with fumonisin than those in the control treatment and the *B. fusca*-infested treatment of the *Bt* hybrid (Table 6). The control treatment of the *Bt* hybrid had significantly lower fumonisin levels than the non-*Bt* isohybrid 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* isohybrid (Table 6).

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

Treatment	Hybrid	
	Bt	Non-*Bt*
Control	2.2 a*	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.

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

Fusarium ear rot and fumonisin production in treatments with *F. verticillioides* and *B. fusca* were lower than in the treatment where Benfuracarb was applied, possibly due to reduced stem borer damage. Benfuracarb application consistently and significantly ($P < 0.05$) reduced Fusarium ear rot (Table 7) and fumonisin production (Table 8) in the 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).

3.3. Meteorological data

The trials in this study were planted during the 2009/10, 2010/11 and 2011/12 seasons. The 2009/10 season had the highest mean rainfall of 137.4 mm and cool conditions with heat units of −110.6, 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. The 2011/12 season had mean rainfall of 74.2 mm and heat units equal to −58.

3.4. Simple regression analyses

A positive correlation was obtained between fumonisin production and Fusarium ear rot ($r = 0.39$), and the quantity of target

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

Treatment	Ear rot (%)
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 that were treated with Benfuracarb and Beta-cyfluthrin to control *Busseola fusca*.

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 that was treated with Benfuracarb and Beta-cyfluthrin to control *Busseola fusca*.

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.

DNA of fumonisin-producing *Fusarium* spp. ($r = 0.63$). There was also a positive correlation between Fusarium ear rot and the quantity of target DNA of fumonisin-producing *Fusarium* spp. ($r = 0.3$) in the *Bt* hybrid (Table 10). In the non-*Bt* isohybrid, the cumulative tunnel length caused by *B. fusca* larvae (*B. fusca* damage) poorly correlated with Fusarium ear rot ($r = 0.23$). 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 the 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 Fusarium ear rot ($r = 0.28$), 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 Fusarium ear rot ($r = 0.47$), and the quantity of target DNA of fumonisin-producing *Fusarium* spp. ($r = 0.46$), as well as between Fusarium ear rot and the quantity of target DNA of fumonisin-producing *Fusarium* spp. ($r = 0.48$) were obtained (Table 10).

4. Discussion

This study demonstrated that *Bt* maize and Benfuracarb have a significant impact on *B. fusca* damage, and under some environmental conditions, it will be associated with reduced Fusarium ear rot development in maize in South Africa. This reduction can be attributed to reduced stem borer damage in *Bt* maize compared to non-*Bt* maize, particularly during the 2011/12 season. Stem borers have previously been reported to be associated with increased Fusarium ear rot (Flett and Van Rensburg, 1992; Munkvold et al., 1997; Bakan et al., 2002), most likely due to the damage they cause to maize kernels that stimulate fungal contamination and discolouration of maize kernels. Wounds created by *B. fusca* can enable infection of maize plants with *F. verticillioides* resulting in the transition from symptomless endophytism to necrotrophic pathogenicity (Rutherford et al., 2002). On the other hand, Ako et al. (2003) showed that certain lepidopteran moths 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 colonisation. 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 colonisation 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 compared to the non-*Bt* hybrid, thereby indicating the effectiveness of the *Bt* toxin against *B. fusca* larvae in this study. 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 South African farming conditions where *B. fusca* is the main stem borer species. 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 indicates that the *Bt* hybrid had no effect on colonisation of maize ears by fumonisin-producing *Fusarium* spp. and subsequent production of fumonisins under high *F. verticillioides* infection pressure. Therefore, the effect of *Bt* maize on fumonisin production in maize ears was indirectly associated

Table 10
Regression analyses in a *Bt*, its non-*Bt* isohybrid and an unrelated non-*Bt* maize hybrid 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

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

with its control of stem borer damage.

Benfuracarb application significantly reduced the levels of Fusarium ear rot and fumonisin production while Beta-cyfluthrin did not, 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). However, this was confounded, since fumonisin production and Fusarium ear rot were correlated. Benfuracarb is also used as nematicide for the control of root-lesion nematodes, *Pratylenchus zeae* Graham and *P. brachyurus* Godfrey, which attack maize in South Africa (McDonald et al., 1987). This might reduce *F. verticillioides* infection through nematode-produced wounding on 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 (Ncube et al., 2017).

Beta-cyfluthrin was applied in the whorls and was not as effective as Benfuracarb in the control of stem borers. *Busseola fusca* damage to maize ears significantly increased compared to the control when no insecticides were 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) larvae but did not affect the *Fusarium* mycoflora. The insecticide itself, 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 indirectly reduces Fusarium ear rot, this was not consistent over seasons. In the 2009/10 season, for instance, no significant difference in Fusarium ear rot was found between *Bt* and non-*Bt* maize. Moreover, Fusarium ear rot development was significantly lower than in the 2010/11 and 2011/12 seasons in the non-*Bt* isohybrid. The low Fusarium ear rot incidence in 2009/10 might have been due to cool and wet conditions, which were not favourable for disease development (Murillo-Williams and Munkvold, 2008; Janse van Rensburg, 2012). Fusarium ear rot development and fumonisin production varied over seasons, indicating the importance of environmental conditions on Fusarium ear rot and fumonisin production. This is in agreement with other studies that indicated that environmental conditions are also important in Fusarium ear rot development and fumonisin production (Barroso et al., 2017; Maschietto et al., 2017). *Busseola 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 (Ncube et al., 2017). This study showed that *Bt* maize and Benfuracarb insecticide application for stem borer control reduced *B. fusca* damage to maize ears and were, therefore, indirectly effective in reducing Fusarium ear rot and fumonisin production. This further suggests that Benfuracarb application is a good management strategy when *B. fusca* and *F. verticillioides* are likely to be co-occurring, particularly in the South African maize production context, where commercial maize production is largely reliant on *Bt* maize for the control of stem borers (James, 2012; Van den Berg et al., 2013). Seasonal variation in Fusarium ear rot and fumonisin production by *F. verticillioides*, however, indicated the importance of climatic conditions on Fusarium ear rot and fumonisin production in maize.

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