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Efficacy of fungal and bacterial antagonists for controlling growth, *FUM1* gene expression and fumonisin B_1 production by *Fusarium verticillioides* on maize cobs of different ripening stages

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Running title: **Microbial antagonists to control growth, gene expression and fumonisin production by *Fusarium verticillioides* on maize cobs**

Key words: biocontrol, *Fusarium*, fumonisin B_1, *FUM* genes, maize cob, maturity stages, silking

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ABSTRACT
This study was carried out to examine the efficacy of two biocontrol agents (*Clonostachys rosea* 016, BCA1; Gram-negative bacterium, BCA5) for control of *FUM1* gene expression and fumonisin B_1 (FB_1) production by *F. verticillioides* FV1 on maize cobs of different ripening stages: R_3, Milk (0.985 a_w); R_4, Dough (0.976 a_w); R_5, Dent (0.958 a_w). Initially, temporal studies on *FUM1* gene expression and FB_1 production were performed on maize kernels for up to 14 days. This revealed that day 10 was optimum for both parameters, and was used in the biocontrol studies. Maize cobs were inoculated with 50:50 mixtures of the pathogen:antagonist inoculum and incubated in environmental chambers to maintain the natural a_w conditions for ten days at 25 and 30°C. The growth rates of *F. verticillioides* FV1, the relative expression of the *FUM1* gene and FB_1 production were quantified. It was found that, $a_w \times$ temp had significant impacts on growth, *FUM1* gene expression and FB_1 production by *F. verticillioides* FV1 on maize cobs of different maturities. The fungal antagonist (BCA1) significantly reduced FB_1 contamination on maize cobs by >70% at 25°C, and almost 60% at 30°C regardless of maize ripening stage. For the bacterial antagonist (BCA5) however, FB_1 levels on maize cobs were significantly decreased only in some treatments. These results suggest that efficacy of antagonists to control mycotoxin production in ripening maize cobs needs to take account of the ecophysiology of the pathogen and the antagonists, as well as the physiological status of the maize during silking to ensure effective control.

1. INTRODUCTION

Fusarium verticillioides (Sacc.) Nirenberg is major pathogen of maize (*Zea mays* L.) of economic importance worldwide (Desjardins, 2006). In the field, *F. verticillioides* is capable of causing systemic infection of maize plant such as seed rot, seedling blight, root, stalk and kernel rot (Bacon *et al*., 2008; Battilani *et al*., 2011). In addition, under suitable environmental conditions, *F. verticillioides* also contaminates ripening maize with the fumonisin group of mycotoxins for which legislative limits in the EU and in many other regions exist (Ahangarkani *et al*., 2014; Chen *et al*., 2014). Fumonisins are class 2B carcinogens; possibly carcinogenic to humans (IARC, 1993), with a provisional maximum tolerable daily intake (PMTDI) of 2 µg/kg body weight as outlined by the WHO (Gareis *et al*., 2003). Of the 15 currently isolated and characterised fumonisins (A-, B-, C-, P-series; Falavigna *et al*., 2012), fumonisin B_1 (FB_1) remains the most abundantly occurring and toxically potent (Stockmann-Juvalla and Savolainen, 2008). Maximum levels of *Fusarium* toxins in maize and maize-based products standardised by the EU are: 4 µg/g total FBs in unprocessed maize, and 1 µg/g total FBs in maize intended for direct human consumption (European Union, 2007). Production of fumonisins by *F. verticillioides* during silking (anthesis) consequently impacts on food and feed quality.

At present, there is significant effort being made to develop control strategies for minimising mycotoxin contamination of staple food crops such as maize by the development of alternative or integrated control strategies less reliant on fungicides. The application of biological control agents (BCAs) using microbial inoculants is being increasingly recognised as a potentially cheaper, viable and eco-friendly alternative that limits the excessive use of synthetic chemical pesticides (Charan *et al*., 2011). However, few studies have focused on controlling the pathogenic phase of *F. verticillioides* to try and control FB_1 contamination, especially on different ripening stages of maize cobs.

Recently, Samsudin and Magan (2016) screened a range of potential BCAs for competition with and controlling growth and FB_1 production by strains of *F. verticillioides*. Control of FB_1 production was influenced by water activity (a_w) and the inoculum ratio of antagonist to pathogen. Two of the best antagonists were a fungal strain (*Clonostachys rosea* 016) and a Gram-negative bacterium. For these two antagonists, the level of control of FB_1 contamination was influenced by a_w, temperature and ratio of antagonist to pathogen in both maize-based media and stored maize grain (Samsudin and Magan, 2016; Samsudin *et al*., 2016). Overall, *C. rosea* 016 gave almost complete control *in vitro* and >80% in stored maize grain. The bacterial antagonist gave a maximum of 75% control, depending on environmental conditions. However, there was a need to examine the efficacy of the BCAs in controlling *F. verticillioides* and FB_1 production at different ripening stages of maize cobs which, in reality, represent different nutritional and a_w levels: R_3, Milk (0.985 a_w); R_4, Dough (0.976 a_w); R_5, Dent (0.958 a_w).

The impact of the BCAs on the key biosynthetic genes involved in the biosynthesis of fumonisins (*FUM* genes) would be a good indicator of efficacy in the different ripening stages of the maize cobs. These *FUM* genes are clustered together (Proctor *et al*., 2003). One of the cluster genes, *FUM1*, encodes for a polyketide synthase (PKS) which is required for fumonisin biosynthesis (Proctor *et al*., 1999; Bojja *et al*., 2004). Previously, expression of *FUM1* and other *FUM* genes have been shown to be a good indicator of the relative production of the toxin, especially in *in vitro* studies (López-Errasquín *et al.*, 2007; Jurado *et al.*, 2008; Lazzaro *et al.*, 2012a, b; Medina *et al.*, 2013). However, the expression of the *FUM* genes as an indicator of *F. verticillioides* infection and relative control by antagonists on different ripening stages of maize cobs has surprisingly not often been examined previously. Recent studies with bacterial antagonists of *Aspergillus flavus* and aflatoxin B_1 control showed that efficacy could be evaluated based on expression of one of the regulatory genes (*aflR*; Al Saad *et al*., 2015).

The objectives of this study were therefore to compare the effects of two BCAs; BCA1 (*C. rosea* 016), BCA5 (Gram-negative bacterium) when inoculated in a 50:50 antagonist:pathogen inoculum ratio in different ripening stages of maize cobs at 25 and 30°C on (a) growth, (b) *FUM1* gene expression and (c) FB_1 production by the pathogen *F. verticillioides* FV1.

2. MATERIALS AND METHODS

2.1 Microorganisms

A FB_1-producing strain of *F. verticillioides* FV1 which was isolated from Malaysian maize kernels and identified morphologically and molecularly; BCA1 (*C. rosea* 016); and BCA5 (Gram-negative bacterium) were used in the present study. BCA1 and BCA5 have been shown to significantly inhibit FB_1 production by *F. verticillioides* FV1 in maize agar and stored maize kernels in previous studies (Samsudin and Magan, 2016; Samsudin *et al*., 2016).

2.2 Temporal studies on *FUM1* gene expression and fumonisin B_1 production by *Fusarium verticillioides* FV1 on maize kernels

Initial experiments were carried out to study the temporal *FUM1* gene expression in relation to fumonisin B_1 production by *F. verticillioides* FV1 on maize kernels. Ten grams

dry gamma-irradiated maize kernels were aseptically transferred into 55 mm Ø Petri plates. The a_w of the dry maize kernels were modified to 0.955 and 0.982 a_w by reference to the moisture adsorption curve for irradiated maize kernels (Samsudin, 2015), and left to equilibrate for 24 hr. The spore inoculum of *F. verticillioides* FV1 was obtained from a 10 day old culture grown on a 3% milled-maize agar at 25°C. Following an equilibration period, a single layer of maize kernels was aseptically inoculated with 1 mL spore inoculum at a concentration of ≈10^6 spore/mL. All experiments were carried out with three replicates per treatment a_w level.

All treatments were incubated at 25°C in separate environmental chambers each containing a beaker of glycerol/water solution (500 mL) to help maintain the equilibrium relative humidity (ERH) of the atmosphere at the target a_w levels of the treatments. After 5, 7, 10 and 14 days incubation, Petri plates containing colonised maize kernels were destructively sampled with half being used for molecular analyses and the other half for FB_1 quantification. The kernel samples for molecular analyses were immediately frozen in liquid nitrogen and stored at -80°C until further use.

(a) *FUM1* gene quantification

For *FUM1* gene expression analysis, four sequential steps were carried out: (1) RNA isolation of *F. verticillioides* FV1 from maize kernels, (2) reverse transcription to convert mRNA into complementary DNA (cDNA synthesis), (3) amplification of *FUM1* gene using qPCR, and (4) absolute quantification of *FUM1* gene expression using the standard curve method.

1. RNA isolation of FV1 from maize kernels

RNeasy® Plant Mini Kit (Qiagen, Germany) was used for RNA isolation following the manufacturer's instruction. The extracted total RNA was stored at -80°C until further analysis. The RNA concentration and purity (A_{260}/A_{280} ratio) were determined spectrophotometrically with a 2.5 µL aliquot on the Picodrop™ (Spectra Services Inc., USA). A ratio between 1.8 and 2.1 is indicative of highly purified RNA (Ahmad-Ganaie and Ali, 2014; Gallagher, 2001).

2. Reverse transcription (cDNA synthesis)

The Omniscript® RT Kit (Qiagen, Germany) was used to synthesise cDNA from 5 µL of total RNA (500 ng) following the manufacturer's instruction. A thermal cycler (Techne™ Thermal cycler TC-312, UK) was used to complete the reaction for 60 min at 37°C followed by 5 min at 93°C. The resulting cDNA were stored at -20°C until further use.

3. Amplification of FUM1 *gene using qPCR*

qPCR was used to quantify the *FUM1* gene expression. The non-specific intercalating dye SYBR® Green (Takara Bio Inc., Japan) was used to detect target amplification. Primer design and optimisation of qPCR conditions were performed before conducting the assay by meeting the criteria proposed by Rodríguez *et al.*, 2015. In this work, the primers PQF1-F (5'-GAGCCGAGTCAGCAAGGATT-3') and PQF1-R (5'-AGGGTTCGTGAGCCAAGGA-3'; López-Errasquín *et al.*, 2007) were used. The concentration of primers and reagents were optimised by selecting the combination which gave the lowest value of quantification cycle (C_q; the cycle at which the fluorescent signal crosses the threshold line or exceeds background) in the amplification plots, and the highest fluorescent signal for a fixed target concentration. Optimal cycling conditions were determined by testing several annealing temperatures from 55 to 60°C.

The Bio-Rad CFX96 Real Time PCR Detection System (Bio-Rad, USA) was used to carry out the qPCR assays. They were prepared in triplicates of 12.5 µL reaction mixture in MicroAmp optical 96-well reaction plates, and sealed with optical adhesive covers (Bio-Rad, USA). Three replicates of an RNA control sample together with a template-free negative control were also included in the runs. The optimal reaction mixture consisted of 6.25 µL SYBR® Green (Takara Bio Inc., Japan), 300 nM of each primer, and 2.5 µL of cDNA template in a final volume of 12.5 µL. The optimal thermal cycling conditions included an initial step of 10 min at 95°C, and 40 cycles of 95°C for 15 sec + 60°C for 30 sec. Data analysis was carried out using the software CFX Manager™ version 3.1 (Bio-Rad, USA).

4. Absolute quantification of FUM1 *gene expression*

A standard curve was generated for absolute quantification of the *FUM1* mRNA copies. To generate the standard curve, a larger PCR fragment of the *FUM1* gene was used as template, using the primers FUM1-Fc (5'-TCTTCAAGGCTCATGGCAGG-3') and FUM1-Rc (5'-CAAGCCGAGTCCAGAGTGTT-3'). The concentration of this standard PCR product was determined by the Picodrop™, and the number of copies was calculated. These stock solutions were diluted serially by a factor of 10 and an aliquot of the dilutions was used as a copy number standard during each setup of the qPCR reaction. The concentration of unknown samples was calculated by the Bio-Rad CFX96 Real Time PCR Detection System (Bio-Rad, USA) according to the generated standard curve. Figure 1 shows the standard curve obtained. The efficiency of the optimised qPCR was checked by the formula; efficiency = $10^{(-1/slope)} - 1$, in which the slope was obtained from the standard curve. In the present work, the qPCR efficiency was 98.82%.

(b) Fumonisin B_1 quantification

1. FB_1 extraction from colonised maize kernels

Following incubation periods, fungal-colonised maize kernels were oven-dried (≈75°C; Genlab, UK) overnight and ground into a fine powder. Next, 10 g ground maize was added to 50 mL extraction solvent (methanol:acetonitrile:water 1:1:2, v/v/v) before shaking (MaxQ 5000; Thermo Scientific, USA) at 300 rpm for 30 min. The mixture was later centrifuged (Labofuge 400 R; Thermo Scientific, USA) for 10 min at 3,500 *xg*, and filtered through Whatman® glass microfiber filters GF/A (90 mm Ø, GE Healthcare, UK). Next, 10 mL extraction filtrate was added to 40 mL phosphate-buffered saline (PBS) and filtered through glass microfiber filter. Finally, 10 mL diluted filtrate (equivalent to 0.4 g original maize kernel) was collected for the clean-up step.

2. Clean-up with immunoaffinity columns

The clean-up step was carried out using the FumoniStar™ Immunoaffinity Columns (Item No. COIAC3000; Romer, Austria) by passing through 10 mL diluted filtrate at a flow rate of ≈1 mL/min following manufacturer's instructions. The elution of bound fumonisins was carried out by passing 3 mL methanol:glacial acetic acid (98:2, v/v) through the column in several small portions (3 x 1.0 mL). The eluate was evaporated to dryness at approximately 60°C before re-dissolving the residue in 500 µL of HPLC-FLD mobile phase. The recovery rate (%) of the columns was obtained within the range of manufacturer's specification by spiking a known concentration of FB_1 and comparing this with the results of HPLC-FLD quantification.

3. FB_1 quantification by HPLC-FLD
Separation, detection and quantification of FB_1 by a reversed phase-HPLC system linked to a fluorescence detector (HPLC-FLD; Agilent, UK) were performed according to Samsudin and Magan (2016) which involved pre-column derivatisation with *ortho*-phthaldialdehyde (OPA; Sigma-Aldrich, USA) with methanol:0.1 M NaH_2PO_4 (77:23, v/v) as the mobile phase. The chromatographic parameters were 335 nm (excitation) and 440 nm (emission) with 30 µL injection volume. The chromatographic data (luminescence unit peak area) were integrated and calculated using a ChemStation software (Agilent, UK). The limit of detection (LOD) and limit of quantification (LOQ) were estimated by using the formulae; LOD = $3\sigma/s$ and LOQ = $10\sigma/s$, in which σ is standard deviation of y-intercepts of the FB_1 linear calibration curve, and s is the slope of the calibration curve.

2.3 Effects of biocontrol agents (BCAs) on *FUM1* expression and FB_1 production by *Fusarium verticillioides* FV1 on maize cobs of different ripening stages
Maize cobs of different ripening stages (R_3: Milk, R_4: Dough, R_5: Dent) were obtained from the NIAB-EMR farm (National Institute of Agriculture and Botany-East Malling Research; Cambridge, UK) in September and October 2012. The type of maize was ES Regain (Euralis Semences; forage maize). Harvested maize cobs were promptly taken back to the laboratory where the a_w was measured (Aqualab, USA) for sub-samples of detached kernels from the entire cob (5-10 maize kernels from the apical, middle and distal parts of the cobs). Maize cobs were then divided into two batches for the two temperature conditions used; 25°C and 30°C.

Spore (or cell) suspensions (≈10^6 spore (or cell)/mL) for FV1, BCA1, and BCA5 were prepared, and inoculated at three points on the maize cobs; (i) 100 µL FV1, (ii) 100 µL FV1 + 100 µL BCA1, and (iii) 100 µL FV1 + 100 µL BCA5. The inoculation was done in triplicates (three separate maize cobs), and placed in separate environmental chambers (25°C and 30°C) each containing a beaker of glycerol/water solution (500 mL) to help maintain the ERH at the target a_w of maize cob treatments.

Inoculated maize cobs (2 temperatures × 3 ripening stages × 3 treatments × 3 replicates) were incubated for ten days following which, 10-15 colonised kernels were removed, labelled, and divided into two equal batches for *FUM1* gene expression studies by RT-qPCR and FB_1 toxin production studies by HPLC-FLD according to the procedures described previously.

2.4 Statistical analysis
Measurements from triplicates were averaged and presented as mean ± SE. Normal distribution of datasets was checked by the Kolmogorov-Smirnov normality test. Analysis of Variance (ANOVA) was applied on normally distributed datasets to analyse the variation between and within group means with 95% confidence interval. $p < 0.05$ was accepted as significant difference. Fisher's Least Significant Difference (LSD) with $\alpha = 0.05$ was applied to compare significant difference between means of treatments using the statistical software Minitab® version 14.0 (Minitab Inc.; Pennsylvania, USA).

3. RESULTS

3.1 Temporal studies on *FUM1* gene expression and fumonisin B_1 production by *Fusarium verticillioides* FV1 on maize kernels

Figure 2 shows the effects of incubation time × a_w on *FUM1* gene expression by *F. verticillioides* FV1 on maize kernels. At 0.95 a_w, there was a significant stimulation in *FUM1* gene expression after 10 and 14 days ($p \leq 0.05$). However, at 0.98 a_w, there was no significant increase in *FUM1* gene expression at any of the sampling times evaluated. The *FUM1* gene expression was higher at 0.98 a_w than 0.95 a_w at all the sampling times.

Figure 3 shows the effects of incubation time × a_w on FB_1 production by *F. verticillioides* FV1 on maize kernels. There was a higher production of FB_1 at 0.95 a_w than 0.98 a_w at all sampling times ($p \leq 0.05$). At 0.95 a_w, FB_1 production was highest after 5 and 10 days incubation. However, at 0.98 a_w, this was highest after 7 and 14 days ($p \leq 0.05$).

Significant interactions ($p \leq 0.05$) between the effects of incubation time × a_w on *FUM1* gene expression and FB_1 production were obtained (Table 1). Positive correlations (Pearson's correlation coefficient, *r*) were also obtained between *FUM1* gene expression and FB_1 production at different incubation time × a_w levels (Table 2).

3.2 Effects of biocontrol agents (BCAs) on *FUM1* expression and FB_1 production by *Fusarium verticillioides* FV1 on maize cobs of different ripening stages

Figure 4 compares the diametric colony development (cm) of FV1, FV1+BCA1 and FV1+BCA5 on maize cobs of different ripening stages (R_3, R_4, R_5) after ten days incubation at 25 and 30°C. There was no significant difference ($p > 0.05$) between treatments across all ripening stages at 25°C. However, at 30°C, no growth was observed at R_3, while sparse growth was observed at R_4. At R_4 and R_5, the colony diameters of all treatments appeared to be significantly ($p \leq 0.05$) smaller at 30°C when compared to 25°C.

Figure 5 shows the effects of treatments at different ripening stages (R_3, R_4, R_5) on *FUM1* gene expression after ten days incubation at 25 and 30°C. At both temperatures tested, across different ripening stages, there was no significant difference ($p > 0.05$) between *F. verticillioides* FV1 and FV1+BCA5 treatments. However, in FV1+BCA1 treatment, there was significant decrease ($p \leq 0.05$) when compared to the control treatments (FV1) at both temperatures. A significant decrease ($p \leq 0.05$) was also observed in the FV1+BCA1 treatments between R_4 and R_5 at both temperatures.

Figure 6 shows the effects of treatments at different ripening stages (R_3, R_4, R_5) on FB_1 production after ten days incubation at 25 and 30°C. Overall, the control treatments (FV1) had the highest FB_1 levels ($p \leq 0.05$) at R_4 and R_5 at 25°C, and at R_4 at 30°C compared to the other treatments. The mixture of FV1 and either BCA1 or BCA5 showed a significant decrease ($p \leq 0.05$) when compared to the control treatments (FV1) at both temperatures tested. It is also noteworthy that while both BCAs were significantly different ($p \leq 0.05$) from each other at R_5 and R_3, there was no significant difference ($p > 0.05$) at R_4 between the treatments at both temperatures tested. In the control treatments (FV1) at both temperatures, an increase in FB_1 production occurred at all

ripening stages at 25°C ($p \leq 0.05$), and there was no significant difference ($p > 0.05$) in FB_1 production between maturity stages R_4 and R_5 at 30°C.
Significant interactions ($p \leq 0.05$) between the effects of temperature × ripening stage on *FUM1* gene expression and FB_1 production were obtained (Table 3). Positive correlations (Pearson's correlation coefficient, *r*) were also obtained between *FUM1* gene expression and FB_1 production at the different temperature × ripening stages examined (Table 4).

4. DISCUSSION

Temporal studies were focused on the relationship between *FUM1* gene expression and FB_1 production on maize kernels at different incubation time × a_w levels. This showed that there were some fluctuations in temporal FB_1 production, regardless of *FUM1* gene expression. This could potentially be due to oxygen depletion during incubation or the breakdown of FB_1 by the fungus itself and subsequent production in a cyclical manner (LeBars *et al*., 1994). Others have suggested that this could be due to enzymatic cleavage of the toxin, or its conversion to other related compounds, or both (Alberts *et al*., 1990). Alternatively, this also could be due to the fact that gene expression normally occurs slightly before mycotoxin production, and that the sampling day for both was the same in the present work. Nevertheless, a strong correlation was obtained between *FUM1* gene expression and FB_1 production at 0.95 a_w (Pearson's $r = 0.8884 \pm 0.13$) and 0.98 a_w (Pearson's $r = 0.8061 \pm 0.13$) at the different sampling times tested. This provided evidence that the *FUM1* gene expression could indeed be used to compare the efficacy of the biocontrol agents at different ripening stages of maize cobs. It is also noteworthy that while no significant difference was observed for *FUM1* gene expression at 0.98 a_w, it was the highest at day 10 and 14 at 0.95 a_w. The corresponding FB_1 production was also highest at day 10. Thus, subsequent studies on different ripening stages of maize cobs were carried out for a 10 day period.

In the maize cobs experiment, three key quantifiable parameters were assessed, *i.e.*, *F. verticillioides* FV1 colony development, *FUM1* gene expression and FB_1 production in the presence of equal inoculums (50:50) of the pathogen and antagonists. In terms of colony development of *F. verticillioides* FV1 on the maize cobs, no significant difference was obtained at 25°C across all three ripening stages. Interestingly, at 30°C no growth was observed at R_3, minimal growth at R_4, and significantly smaller colony diameters at the R_5 stage when compared to 25°C. This might suggest that 30°C may not be the optimum temperature for *F. verticillioides* FV1 colonisation of maize cobs. This contrasts with previous studies which suggested that on milled-maize agar and layers of harvested maize kernels, there was little difference between growth at 25 and 30°C, regardless of a_w levels (Marín *et al*., 2004; Samsudin and Magan, 2016). Perhaps in natural agro-systems, drier and warmer conditions favour the colonisation by *Fusarium* spp. during the grain filling stage (R_1 silking to R_6 maturity) that leads to *Fusarium* kernel rot (Venturini *et al*., 2015; Miller, 2001). However, information on the extent of colonisation in relation to a_w and temperature in each of the maize ripening stages has not been fully documented previously. As demonstrated in the present work, the colony development of *F. verticillioides* FV1 in the control treatments was always more rapid at 25°C across all ripening stages examined although not significantly different. It was also observed that as a_w decreased across the ripening stages at 25°C, the colonisation rate increased in all the treatments although there was no significant different between the aw

treatments. This suggests that the optimum a_w for colonisation of maize cobs by *F. verticillioides* FV1 is wider than was previously thought. Overall, temperature appeared to play a bigger role in determining colonisation than the actual a_w of the maize cobs in the present work.

It was noticeable that the bacterial candidate (BCA5) appeared to have no effect on growth of *F. verticillioides* FV1. This might suggest that while bacterial biocontrol agents of maize pathogens are frequently applied as soil or seed treatments (Cavaglieri *et al.*, 2005; Bacon *et al.*, 2001; Bacon and Hinton, 2000) with some inhibitory effects, application to maize cobs yielded lower efficacy. This might due to the fact that bacteria require freely available water for multiplication, and damage to the maize kernels may also be critical for efficacy. In contrast with the fungal candidate (BCA1), the colony development appeared to be mainly that of the BCA1 than of *F. verticillioides* FV1. BCA1 seemed to exhibit good potential in outcompeting the pathogen by occupying the niche under similar a_w and temperature conditions, supported by the indication of its visible velvety-white hyphal growth on the maize cobs as compared to the salmon-coloured cotton-like growth of the pathogen in the control treatments. Since this antagonist is well-known as a soil-dwelling mycoparasite and saprophyte (Palazzini *et al.*, 2013; Rodríguez *et al.*, 2011; Mejía *et al.*, 2008), it may also be effective in soil-based treatments to reduce *F. verticillioides* inoculum potential on crop residue or in soil.

The critical aspect is the ability of the antagonists to control FB_1 contamination of the maize cobs of different ripening stages. Thus, the effects on *FUM1* gene expression and FB_1 production were important. The present work demonstrated that at 25°C, *FUM1* gene was expressed in higher amounts (log_{10} 1.4 – 4.3) when compared with 30°C (log_{10} 1.3 – 3.1). This indicates some effects of temperature on *FUM1* gene expression by *F. verticillioides* FV1. Overall, *FUM1* gene expression in the control treatments, irrespective of ripening stages at both temperatures, was not significantly different. This indicated that the a_w range examined in ripening maize cobs had no significant effect on *FUM1* gene expression. This in turn is indicative of the ability of *F. verticillioides* FV1 to effectively produce FB_1 over a range of ripening conditions if it becomes established.

The bacterial antagonist (BCA5) had little effect on *FUM1* gene expression at all temperatures and ripening stages tested. In contrast, the fungal antagonist (BCA1) showed significantly lower *FUM1* gene expression at all temperatures and ripening stages tested. This down-regulation of the *FUM1* gene expression confirms that there was an effect of BCA1 on the potential ability of the pathogen to produce fumonisins. This was especially so at 25°C. The slightly lower inhibition in *FUM1* expression at 30°C might be because BCA1 does not grow as well at this temperature.

For FB_1 production, as a_w decreased across the ripening stages at 25°C, toxin levels steadily and significantly increased in the control treatments. Similar increasing production patterns were also observed at 30°C but with no significant difference between R_4 and R_5 (no growth was detected at R_3). These findings were in agreement with previous studies which suggested that under environmental stress, often higher amounts of FB_1 is produced by *F. verticillioides* in naturally contaminated maize crops (Shephard *et al.*, 1996; Fandohan *et al.*, 2003; Bigirwa *et al.*, 2007; Mukanga *et al.*, 2010).

Overall, BCA1 (*C. rosea* 016) was able to reduce FB_1 production in all the maize ripening stages and both temperatures examined. This decrease in FB_1 was 73.1 ± 3.9% at 25°C, and 58.4 ± 4.1% at 30°C when compared to the controls. For the bacterial antagonist BCA5 (Gram-negative bacterium), there was no significant difference between the controls in terms of *F. verticillioides* colony development and *FUM1* gene expression. However, there was an effect on FB_1 production in some cases (25°C+R_4 and R_5; 30°C+R_4). In this case, the inhibition levels were 46.1 ± 9.1% at 25°C, and 22.3 ± 9.3% at 30°C. It may be that BCA5, while not affecting growth of the pathogen, has a physiological effect on the biosynthetic pathway of fumonisins, thus impacting on the phenotypic reductions observed.

CONCLUSIONS

The *FUM1* gene expression can be effectively used in the maize system to examine the potential for contamination with FB_1 based on the strong correlations obtained between *FUM1* expression and FB_1 production on maize kernels and maize cobs at different a_w levels.

This was the first detailed study to compare the efficacy of two biocontrol agents on different ripening stages of maize which represented different a_w levels in maize cobs during ripening in the field. Overall, the fungal biocontrol agent (BCA1; *C. rosea* 016) significantly inhibited FB_1 levels on maize cobs by >70% at 25°C, and almost 60% at 30°C irrespective of maize ripening stages. In contrast, the bacterial biocontrol agent (BCA5; Gram-negative bacterium) was less effective, only reducing FB_1 levels by almost 50% at 25°C, and about 20% at 30°C. Furthermore, BCA5 did not appear to effectively colonise the maize cobs, which might have led to the inefficient control of *FUM1* gene expression and subsequently the FB_1 production. Thus, the mechanism of action of the fungal and bacterial antagonists needs more investigation. However, potential does exist to use *C. rosea* 016 for control of FB_1 contamination of ripening maize but timing of applications of the antagonist is critical for success. More studies are required on production and formulation of such biocontrol agents for improving the potential success either alone or as part of an integrated control strategy.

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TABLES

Table 1. p-values for the effects of incubation time × a_w, and their interactions on *FUM1* gene expression (log_{10} copy number) and fumonisin B_1 production (μg/g maize kernel) as analysed by Analysis of Variance (ANOVA).

		FUM1	**FB_1**
Source of variation	**df[1]**	**p-value**	
Between groups[2] (a_w)	1	0.0000*	0.0000*
Within groups[3] (incubation time)	3	0.0000*	0.0287*
Interaction	3	0.0038*	0.0000*

[1]Degrees of freedom. [2]Number of water activity (2) minus 1. [3]Number of incubation time (4) minus 1. *Significant at p ≤ 0.05.

Table 2. Pearson's correlation coefficient (r) for the correlations between *FUM1* gene expression (log_{10} copy number) and fumonisin B_1 production (μg/g maize kernel) at different incubation time × a_w.

	0.95 a_w	**0.98 a_w**
Day 5	0.9449	0.9256
Day 7	0.9439	0.6617
Day 10	0.9635	0.7274
Day 14	0.7010	0.9097

Table 3. *p*-values for the effects of temperature × cob ripening stage, and their interactions on *FUM1* gene expression (log_{10} copy number) and fumonisin B_1 production (μg/g maize kernel) as analysed by Analysis of Variance (ANOVA).

		FUM1	**FB_1**
Source of variation	**df[1]**	***p*-value**	
Between groups[2] (temperature)	1	0.0000*	0.0004*
Within groups[3] (ripening stage)	2	0.0000*	0.0000*
Interaction	2	0.0000*	0.0000*

[1]Degrees of freedom. [2]Number of temperature (2) minus 1. [3]Number of ripening stage (3) minus 1. *Significant at p ≤ 0.05.

Table 4. Pearson's correlation coefficient (*r*) for the correlations between *FUM1* gene expression (log_{10} copy number) and fumonisin B_1 production (μg/g maize kernel) at different temperature × cob ripening stage.

	25°C	**30°C**
R_3	0.8985	**no growth*
R_4	0.7025	0.6766
R_5	0.8608	0.9207

FIGURE LEGENDS

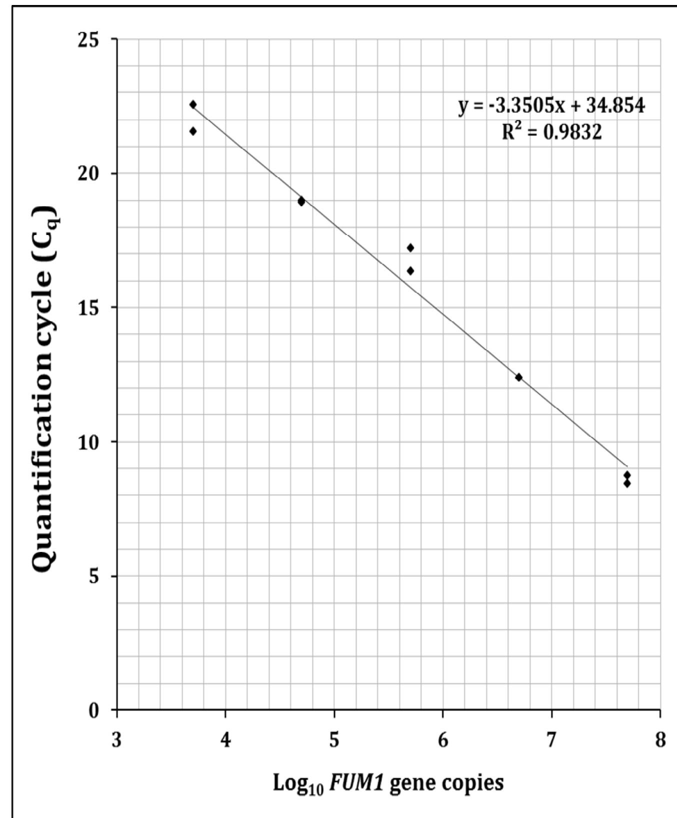

Figure 1. Standard curve for the absolute quantification of *FUM1* gene expression.

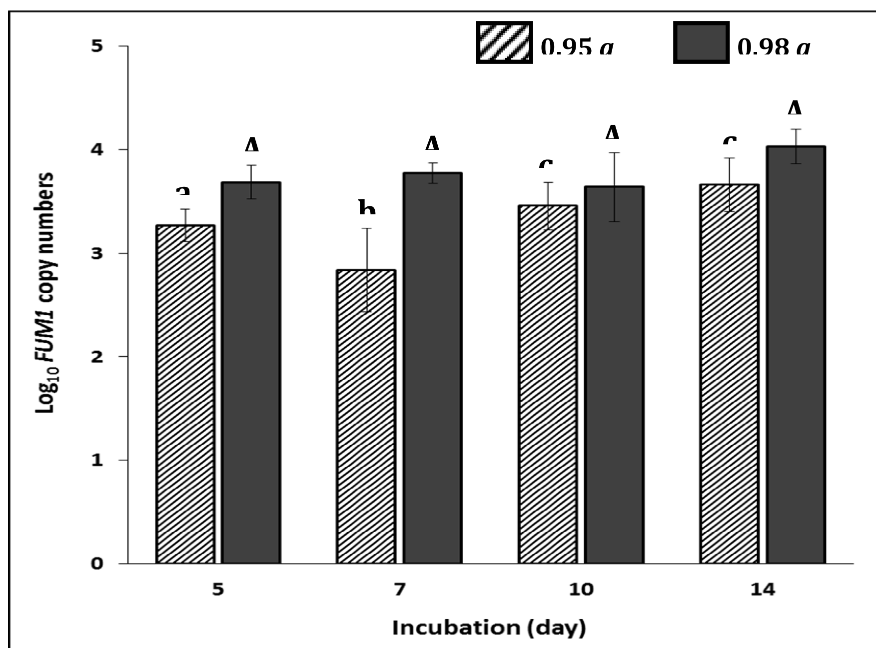

Figure 2. *FUM1* gene expression by *Fusarium verticillioides* FV1 on maize kernels at 0.95 and 0.98 a_w after 5, 7, 10 and 14 days incubation at 25°C. Data are means of replicates (n = 3) with bars indicating SE. Different letters indicate significant difference ($p \leq 0.05$) by Fisher's Least Significant Difference (LSD). Amplification cycle = 40. Log_{10} 1 = 10 copy numbers.

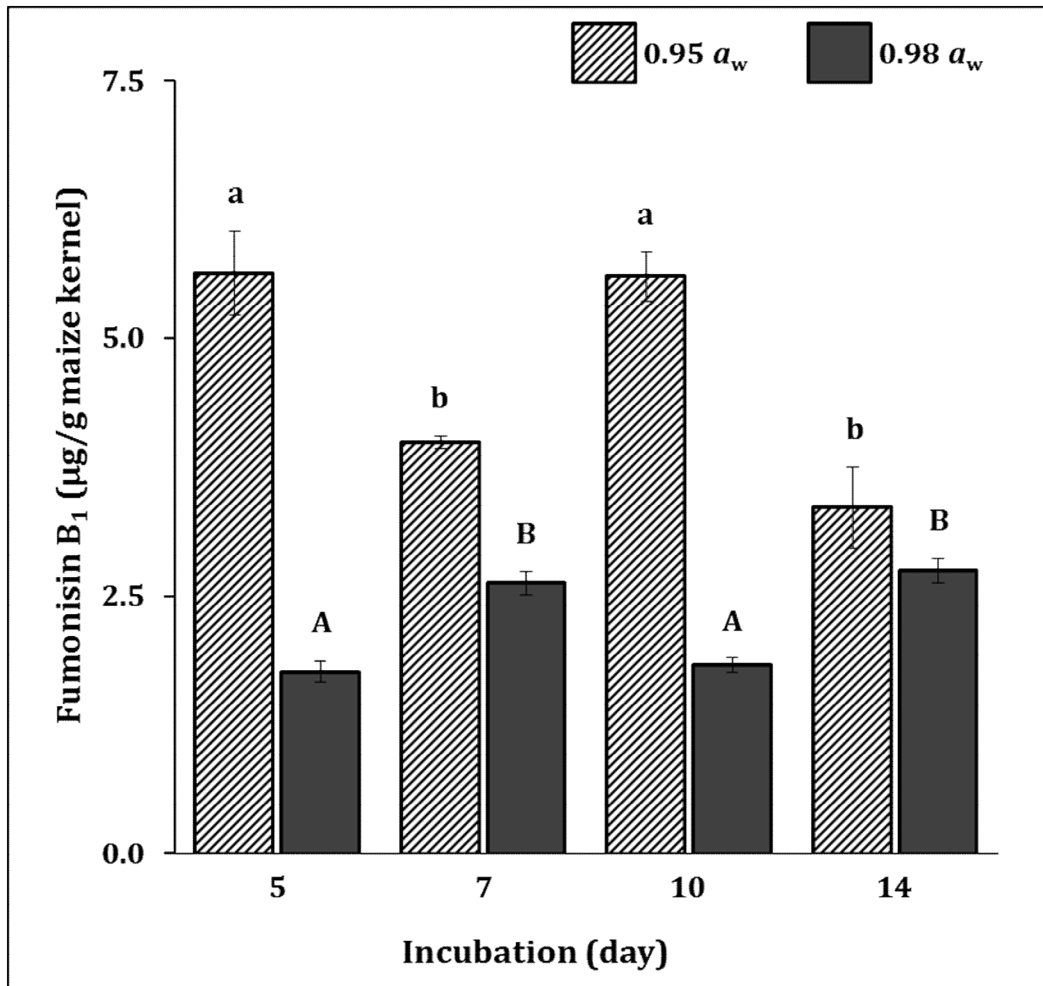

Figure 3. Fumonisin B_1 production by *Fusarium verticillioides* FV1 on maize kernels at 0.95 and 0.98 a_w after 5, 7, 10 and 14 days incubation at 25°C. Data are means of replicates (n = 3) with bars indicating SE. Different letters indicate significant difference ($p \leq 0.05$) by Fisher's Least Significant Difference (LSD). Amplification cycle = 40. Log_{10} 1 = 10 copy numbers.

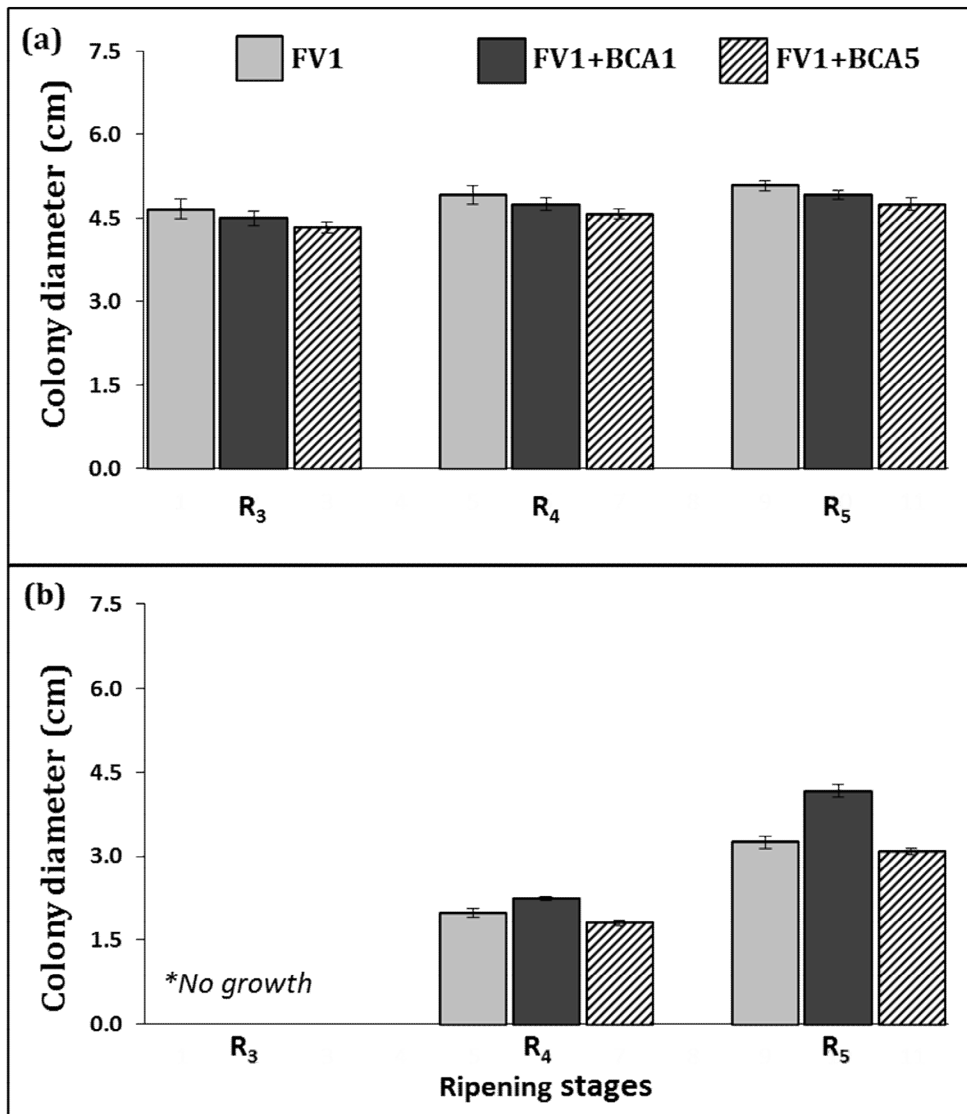

Figure 4. Diametric colonisation (cm) by FV1, FV1+BCA1 and FV1+BCA5 on maize cobs of different ripening stages (R_3, R_4, R_5) after 10 days incubation at (a) 25°C and (b) 30°C. Data are means of replicates with bars indicating SE.

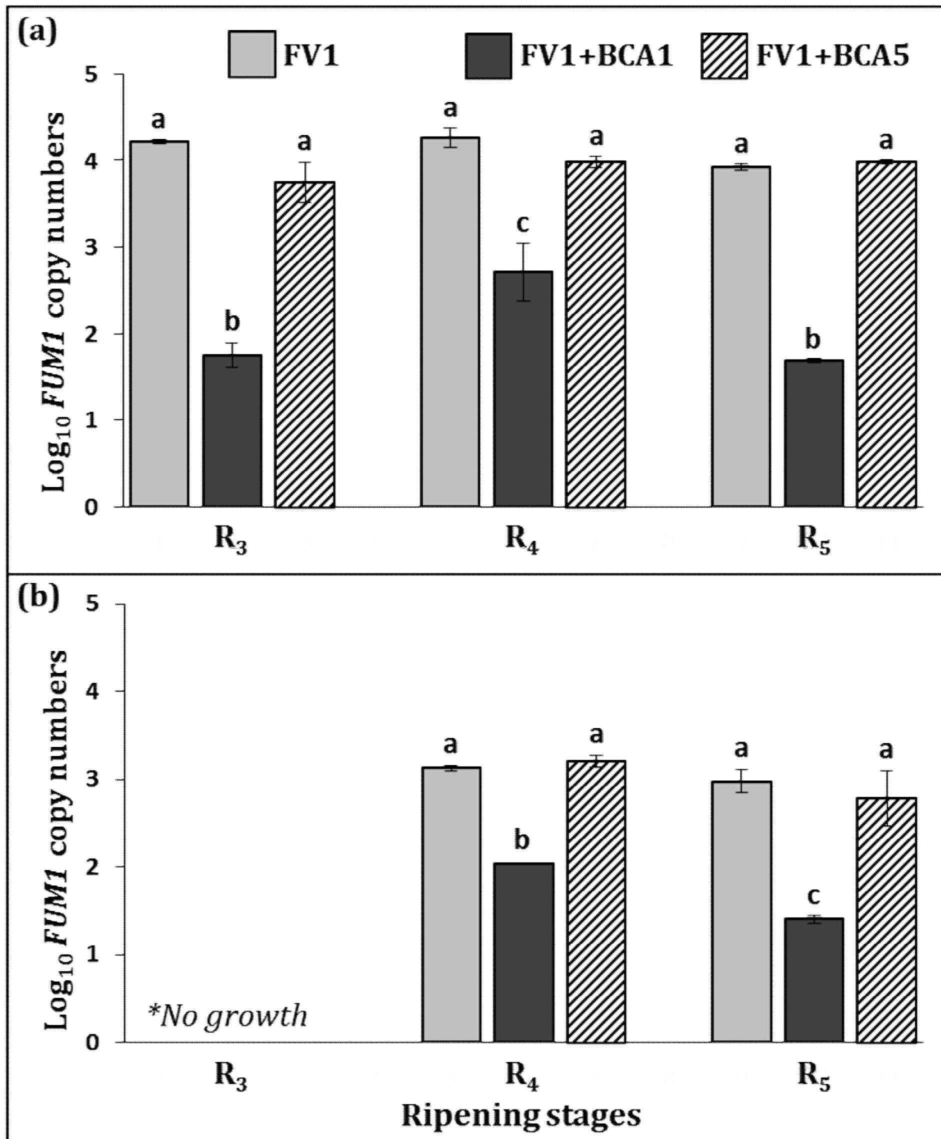

Figure 5. *FUM1* gene expression by FV1, FV1+BCA1 and FV1+BCA5 on maize cobs of different ripening stages (R_3, R_4, R_5) after 10 days incubation at (a) 25°C and (b) 30°C. Data are means of replicates with bars indicating SE. Different letters indicate significant difference ($p \leq 0.05$) by Fisher's Least Significant Difference (LSD). Amplification cycle = 40. Log_{10} 1 = 10 copy numbers.

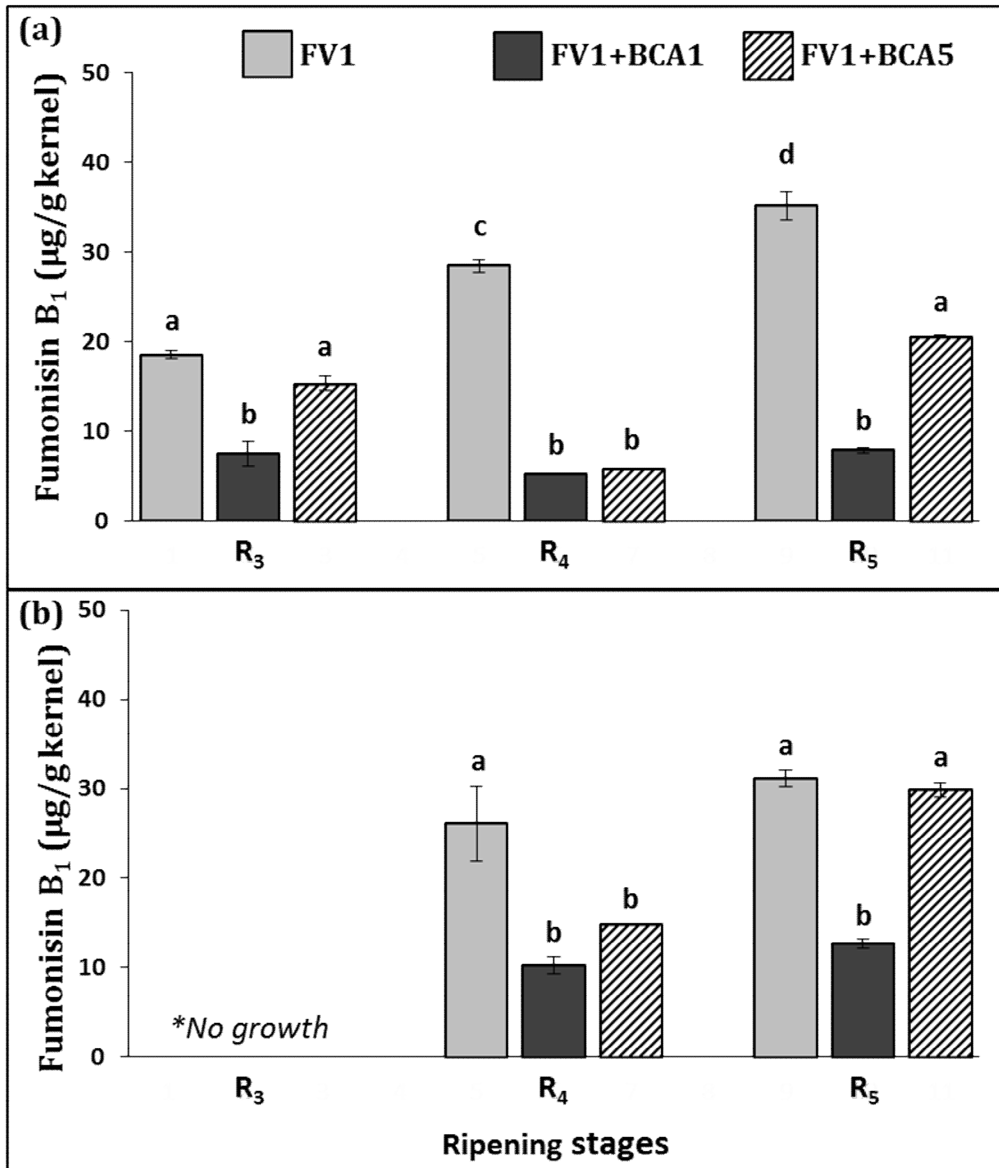

Figure 6. Fumonisin B_1 production by FV1, FV1+BCA1 and FV1+BCA5 on maize cobs of different ripening stages (R3, R4, R5) after 10 days incubation at (a) 25°C and (b) 30°C. Data are means of triplicates. Bars are SEs. Different letters indicate significant difference ($p \leq 0.05$) by Fisher's Least Significant Difference (LSD).

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