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Biocontrol of mycotoxins: dynamics and mechanisms of action

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Abstract
This paper discusses the relationship between biocontrol agents (BCAs) and mycotoxigenic fungi and mycotoxin control. In most cases BCAs are examined for control of growth of fungal pathogens and disease symptoms. However, for mycotoxin control the approach and focus needs to be different. The mechanism of action and the inoculum dose necessary for control of toxin production by *Aspegillus*, *Penicillium* and *Fusarium* species may be different from that for traditional fungal plant pathogens. The mechanisms of action, the relative inoculum potential and the impact that interacting environmental conditions have on control of key components of the life cycle of mycotoxigenic fungi are considered. The practical aspects of production and formulation hurdles are discussed and potential future approaches and strategies which may need to be considered for more effective biocontrol of mycotoxigenic fungi and mycotoxins are presented.

1. Introduction
There has been a lot of impetus in the development of biocontrol agents (BCAs) for the control of fungal diseases and pests in the last decade, driven predominantly by the withdrawal of a range of crop protection chemical structural compound groups by the European Union. Thus, the agrochemical industry has refocused priorities on integrated control to include the use of BCAs in research and programmes for disease and pest control, both pre- and post-harvest.

There has been a significant focus for many years on the use of BCAs for the control of fungal pathogens of a range of durable and horticultural crops for both soil-borne and foliar diseases and indeed weeds. The mechanism of action has often included direct antagonism between

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the BCA and fungal pathogen, competitive exclusion of the pathogen by niche occupation, the production of secondary metabolites, hyperparasitism, and the production of volatile organic compounds (VOCs). In almost all these cases the target has been to reduce plant disease symptoms and reduce yield and quality loss. Plant disease management strategies using BCAs has not included concern about potential contamination of the harvested product with toxic secondary metabolites.

Mycotoxins are fungal secondary metabolites that exhibit toxic effects on both humans and animals. Although >400 different molecules have been described, only around 20 mycotoxins are considered of economic importance, because of their levels of contamination in food and feed products. Mycotoxigenic fungi are either true pathogens (e.g. *Fusarium* species) or secondary pathogens or saprophytes and effective secondary colonisers (e.g. *Aspergillus* and *Penicillium* species) of a range of commodities from cereals to tree nuts to coffee and cocoa and pome fruit. Management of reduction or minimisation strategies for mycotoxin contamination have some similarities and some differences from that for plant disease management. The use of BCAs for the control of mycotoxigenic fungi has focused on (a) efficacy in terms of control of germination/growth/colonisation by mycotoxigenic fungi on/in raw or processed food commodities and (b) reduction in the production of the associated mycotoxin by often targeting the biosynthetic genes involved in toxin biosynthesis. The latter is important as legislation in both the EU and in many other countries world-wide have strict limits on maximum contamination in both raw commodities and processed foods. A key driver to minimise/prevent mycotoxin contamination of food is to prevent or minimise consumer exposure to these toxic compounds.
Glare et al. [1] suggested that the outlook for biopesticides had changed positively and significant market penetration has thus been made for some formulated BCAs although the niche size of the market is still relatively small compared to that of the chemical pesticides. Thus, technologies for transformational step changes in BCA production and formulation are necessary for increasing the uptake and utilisation of BCAs by the agri/horticulture industries.

Screening for identification of BCAs has been carried out for many years and the blueprint for development is probably best exemplified in the excellent review of Kohl et al. [2]. This provides the systematic approaches necessary for commercialisation of both bacterial and fungal BCAs. However, development of BCAs for control of mycotoxigenic moulds and mycotoxin reduction/minimisation may require a slightly different approach. This is because many mycotoxigenic species, under environmental stress, may not colonise a host effectively, but may still be induced to produce more mycotoxins [3, 4]. Thus, the development of BCAs for control of mycotoxins needs to consider (a) the life cycle of the mycotoxigenic species, (b) the fluxes in environmental conditions, (c) the plant agronomy, and (d) interactions with pests, which often provide entry points for mycotoxigenic fungi, especially during the ripening stages (e.g., cereals) or during post-harvest processing (coffee, cocoa and some nuts).

2. Screening of BCAs for control of mycotoxins: environmental considerations

It is critical that when examining potential BCAs for control of mycotoxins that we consider effects on control of colonisation of the commodity by the mycotoxigenic species and the

ability to inhibit/reduce biosynthesis of the mycotoxin. The biosynthetic genes for mycotoxin production are often clustered together and approaches have included the potential for inhibiting specific regulatory or structural genes to reduce or inhibit mycotoxin production.

Macroscopic interactions *in vitro* in relation to environmental conditions: use has been made of a numerical scoring system to identify inhibition under different environmental and nutritional conditions. This has enabled an Index of Dominance to be developed which can be useful to identify those microorganisms which can inhibit/competitively exclude a pathogen and the environmental envelope over which efficacy occurs at a macroscopic level [5, 6]. However, this has been shown to be in a state of flux depending water activity (a_w) x temperature x pH factors and nutritional status [7]. This of course does not provide evidence on whether the production of the relevant mycotoxin is being minimised [8]. Recent studies have utilised a mixed inoculum approach *in vitro* and *in situ* under different interacting environmental conditions to identify directly whether mycotoxin production is being inhibited. The approach has used different ratios of cells/spores of BCA:Mycotoxigenic species (100:0; 75:25; 50:50; 25:75; 0:100) which are spread-plated onto media relevant to the commodity (e.g. 2-3% maize or wheat agar). This has the advantage by providing relevant data on (a) quantification of whether mycotoxin production has been minimised, (b) potential threshold inoculum levels of the BCA necessary for mycotoxin control and (c) use of the fungal biomass to confirm, by using reverse transcription real-time PCR (RT-q-PCR), effects on specific structural or regulatory genes involved in biosynthesis of the target mycotoxin/s. These types of studies have been carried out under different simulated temperature x a_w interacting conditions relevant to the target mycotoxigenic species. Recent studies to control aflatoxin B_1 (AFB_1) production by *Aspergillus flavus* using BCAs including atoxigenic strains of *A. flavus*, and of fumonisin B_1 (FB_1) by *Fusarium verticillioides* have used this approach [6, 9, 10, 11]. This has been complimented in the case of AFB_1 control by examining the effects on relative expression of biosynthetic genes such as *aflD*, *aflM* (structural) and *aflR/aflS* (regulatory) involved in AFB_1 production and the *FUM1* gene as an indication of FB_1 control. This facilitates the identification of those microbial BCA strains which are able to significantly inhibit mycotoxin production and confirm this by quantifying effects on relevant biosynthetic genes under different a_w x temperature conditions relevant to the pathogen [10, 12]. It may be important to balance the control of toxin production by atoxigenic strains and its relative impact on yield and grain quality.

These types of studies also provide useful information and guidance on whether the BCA inoculum requirement for control will be at a threshold level which would be economic to produce and formulate during downstream processing. This is an important consideration when identifying promising candidate BCAs, especially for mycotoxin control. There are also cases where these types of studies highlight that interactions between BCAs and a target mycotoxigenic species can lead to a stimulation of mycotoxin. Thus, Al-Saad et al. [12] found that when screening potential bacterial antagonists for AFB_1 control, 50:50 mixed populations of cells/spores of the antagonist:pathogen resulted in some bacteria stimulated *aflD* and AFB_1 production. This suggests that there may indeed be some signalling or trigger for the mycotoxigenic species to increase the production of secondary metabolites when under abiotic stress. This could be operating similarly to bacterial interactions where quorum sensing (production of homoserine lactones) is considered an important trigger for inhibition of certain interacting species. It is possible that such compounds could be produced during

interactions which contribute to the enhanced production of AFB_1. Volatile compounds are also produced by fungi and the role of these on mycotoxin production should not be neglected. It has been shown that volatile production patterns may be different in strains of species which are able to produce mycotoxins and those which cannot, then it may be possible that the interactions are more complex and other mechanism of action may operate [13, 14].

When examining utilisation of combinations of BCAs with fungicides, special attention has to be paid to the relationship of the pathogen with sub-lethal doses of fungicides. Where, recommended rates of application of fungicides are not used (sub-optimal concentrations) this has sometimes resulted in a stimulation of mycotoxoin production, especially under environmental stress conditions [15, 16, 17].

3. Mechanisms of action

Originally Cotty and Baymans [18] suggested that an atoxigenic strain was able to compete effectively at the same inoculum ratio (50:50) or with even less atoxigenic inoculum on cotton balls or in liquid fermentation systems. They suggested that atoxigenic strains used two mechanisms of action; by exclusion of the toxigenic strain from the niche, and by competing for nutrients destine for aflatoxin biosynthesis. Probably the mechanism of action is a combination of attributes which enables a BCA to effectively compete with a mycotoxigenic species in terms of niche exclusion, or replacement in a niche, as well as the ability to reduce biosynthetic toxin pathways involved in metabolite production. This has to be seen in the context of whether the BCA and the mycotoxigenic species are competing for primary resources or whether the mechanism is via secondary resource capture. True mycotoxigenic pathogens (e.g. *Fusarium graminearum*, *F. verticillioides*) are involved in primary resource capture and effective establishment is thus critical for infection [19]. For secondary pathogens and saprophytes such as *Aspergillus* section *Flavi*, Section *Circumdati* and Section *Nigri* species, which are more xerophilic and able to colonise niches where there is less competition, often pest damage or drought stressed hosts are conducive to colonisation. The BCA needs to be able to effectively compete under such environmental regimes. Abbas et al. [19] suggested that exploitative competition is insufficient to explain the reductions obtained in controlling AFB_1 production by toxigenic strains of *A. flavus*. They suggested that perhaps the host plant may be stimulated to synthesize volatile aldehydes known to suppress AFB_1 production when being colonised by *A. flavus*. The atoxigenic BCA may also be able to produce substances which can repress AFB_1 production during competition with the toxigenic strain, or may induce plant-mediated degradation or perhaps transformation of aflatoxins. We know little about how the concentrations of masked mycotoxins might be impacted by BCAs. No studies have examined this aspect previously.

If we consider the life cycle of *A. flavus* on raw cereal commodities such as maize, then the key areas would be niche exclusion during survival as sclerotia in soil and/or on crop residue or conidial production where BCAs may be able to reduce inoculum potential of *A. flavus*; or during silking when atoxigenic strains or other antagonistic BCAs may be able to colonise the silks and prevent the toxigenic *A. flavus* strains from occupying the ripening maize cob niche.

Few studies have been carried out to understand the environmental influence on sporulation of *A. flavus* (sclerotia and conidia) and practically none to assess the effect of interactions between BCAS and *A. flavus* on relative sporulation of the latter species. Rodriguez-Sixtos [11] examined such interactions on conidial sporulation of the mycotoxigenic pathogen on senescent maize leaves modified to 0.98 a_w (=-2.8 MPa water potential; assayed after 3, 8 days) or 0.93 a_w (=-7.0 MPa water potential; assayed after 8 days) with BCA:pathogen applied in a 50:50 ratio. Figure 1 shows that there was very little effect of any BCAs, including an atoxigenic *A. flavus* strain, on the conidial sporulation of the toxigenic strain of *A. flavus*, regardless of senescent maize leaf a_w condition at 30°C. This certainly suggests that the potential for reducing toxigenic *A. flavus* inoculum potential on crop debris may be difficult to achieve. This needs to be tested in crop debris of other commodities to see whether it is also applicable in terms of reduction of inoculum potential of mycotoxigenic species in general on crop debris by BCAs.

Niche exclusion and C-source utilisation patterns have been considered important mechanism of action. The question arises as to whether this also applies to control of mycotoxigenic fungi. Originally, Wilson and Lindow [20] working with bacterial BCAs to control ice nucleation bacterial pathogens examined the relative utilisation patterns of C-sources in the plant matrix by the pathogen and the candidate BCAs to identify the best one and to better understand the mechanisms of action. By identifying those C-sources utilised in common and those which were utilised by the pathogen, and BCA only, it was possible to develop a Niche Overlap Index (NOI). They suggested that NOI >0.9 indicated occupation of the same niche, and NOI of <0.90 indicated occupation of separate niches, and thus nutritional partitioning. Subsequently, environmental conditions were shown to significantly influence patterns of C-source utilisation and the NOI, especially a_w, temperature and presence or absence of inhibitory compounds [21, 22]. More recently, the NOI and the temporal rate of C-source utilisation patterns (Temporal Carbon Utilisation Sequence, TCUS) were examined for *A. flavus* and other mycotoxigenic species [23], between toxigenic and atoxigenic strains of *A. flavus* [24], and between *F. verticillioides* and the BCA *Clonostachys rosea* [25]. For *F. verticillioides* and *C. rosea* the dominant maize-based C-sources utilised by the pathogen and the antagonist were slightly different when examining effects at 0.995, 0.98 and 0.95 a_w and 25/30°C on patterns of C-source utilisation. Thus, these species appeared to have differential utilisation patterns when occupying the niche suggesting that niche exclusion under some conditions as a factor. The TCUS showed that the *F. verticillioides* strain utilised carbohydrates rapidly followed by amino acids and then one fatty acid, palmitic acid. The antagonist, *C. rosea,* utilised both carbohydrates and amino acids at a similar rate but more slowly than the pathogen. There were also differences in the utilisation of some individual amino acids and carbohydrates. This probably contributed to the occupation of different niches under some interacting environmental conditions. Thus the *C. rosea* may prevent *F. verticillioides* from occupying microniches by utilising certain nutrients faster than the pathogen. Thus, the mechanism of action could include niche exclusion or rate of utilisation of nutritional sources, influencing the ability of the mycotoxigenic species to effectively populate the niche. This may also be linked to the capability for production of relevant hydrolytic enzymes to facilitate occupation under different environmental conditions.

Previously it was shown that a_w x temperature influences the production of hydrolytic enzymes by toxigenic *A. flavus* strains and *F. verticillioides* [12, 26]. Mohale [8, 27] compared the ability of atoxigenic and toxigenic strains of *A. flavus* to produce different hydrolytic enzymes which are critical for niche occupation. Figure 2 compares the hydrolytic enzyme profiles of a toxigenic and atoxigenic strain of *A. flavus*. The atoxigenic strain produced a slightly narrower range of enzymes when compared to the toxigenic strain. More detailed analysis of specific activity of esterase and acid phosphatase using p-nitrophenyl substrates showed that over period of up to 120 hrs there were no significant differences between atoxigenic and toxigenic species [8].

Sultan and Magan [5] showed that secondary metabolites from a *Streptomyces* (AS1) strain isolated from peanuts were very effective at inhibiting growth and AFB_1 production by a toxigenic *A. flavus* strain *in vitro* and *in situ* in stored peanuts. Indeed, regardless of environmental conditions the AS1 metabolites were more effective than when using the bacterial cells as an inoculum, regardless of the concentration of the BCA. More recently, Verheecke et al. [28, 29] examined the interaction between *Streptomyces* strains and both *A. flavus* and *A. parasiticus*. They used RT-q-PCR to try and examine the mechanism of action to quantify effects on five biosynthetic genes (*aflD, aflM, aflP, aflR* and *aflS*). This showed that the *Streptomyces* strains repressed gene expression to a greater level in *A. parasiticus* than in *A. flavus*. Expression of the regulatory genes *aflR* and *aflS* were generally repressed in both species. Expression of *aflM* gene was repressed and correlated with AFB_1 inhibition. These results suggested that *aflM* expression could be a potential indicator of inhibition/control of AFB_1 production. However, these studies did not examine impacts of environmental stress which would have been interesting [see 11].

Recently, studies were carried out with different ripening stages of maize cobs to examine relative efficacy of a fungal and bacterial BCA for FB_1 control [9]. This demonstrated that the ripening stage a_w of the kernels in the cob changes from R_3, Milk (0.985 a_w) to R_4, Dough (0.976 a_w) and R_5, Dent (0.958 a_w). These are within the environmental a_w range for colonisation by *F. verticillioides*. The *C. rosea* strain significantly reduced FB_1 contamination of the maize cobs by >70% at 25°C, and almost 60% at 30°C regardless of maize ripening stage (Figure 3). For the bacterial antagonist, FB_1 levels on maize cobs were significantly decreased only in some treatments, perhaps due to the requirement for freely available water. This was confirmed by examining effects of these conditions on the *FUM1* gene, a key gene in the fumonisin biosynthetic pathway.

Studies on patulin control in pome fruits have been a focus for biocontrol, especially using different yeasts [30]. Yan et al. [31] suggested that biocontrol by the yeast *Rhodotorula mucilaginosa* was enhanced by the addition of phytic acid in treatments to decrease both *Penicillium expansum* infection and patulin production. They also suggested that the yeast + phytic acid could degrade patulin *in vitro*. Indeed, studies have also focused on degradation of AFB_1 by microorganisms, however, this approach in relation to the mycotoxin only, and often unrelated to the plant/mycotoxigenic fungus interface and is then a biotechnology/fermentation approach which is not relevant to biocontrol strategies *per se*, pre- or post-harvest [32].

4. Biocontrol agents and mycotoxin control in practice

Production, formulation and targeting, and timing of the application of BCAs to control growth and inhibit mycotoxin contamination of staple commodities is the critical step for successful minimisation strategies. The use of mixtures of atoxigenic *A. flavus* strains have been successfully utilised in the USA in cotton (Afla-Guard®) and maize (K49) and in West and East Africa in maize and groundnuts (Aflasafe™) to reduce AFB_1 contamination [33]. These BCAs are formulated on colonised sorghum grain as the solid substrate which is then sprinkled on the soil surface to outcompete toxigenic strains. This provides a food base from which the atoxigenic strains can become established and over time dominate the ecological niche.. In both cases, the atoxigenic strains were chosen based on the deletion of key biosynthetic genes or the whole gene cluster to ensure stability and no possibility of AFB_1 production.

Water-soluble formulations [34, 35] with different sprayable formulations of an atoxigenic strains (Afla-Guard®; K49) as well as bioplastics based inoculum for both in soil and on ripening maize cobs during silking have been done. They found >97% reduction of AFB_1 in maize ears and about 65% in soil applications. Recent studies with unformulated *C. rosea* 016 conidia inoculated in a 50:50 ratio with those of *F. verticillioides* in different ripening stages of maize cobs suggested effective control at the R4 and R5 ripening stages only (Figure 3).

A range of native bacteria, yeasts and filamentous fungi have been evaluated as BCAs in field trials including *Bacillus subtilis* and *Brevibacillus* sp. which were effective at reducing deoxynivalenol accumulation in wheat [36]. The application of *C. rosea* isolates on wheat stubble reduced *Fusarium* colonisation on crop debris. *Kluyveromyces thermotolerans* strains have shown potential as BCAs for reducing the inoculum load of *Aspergillus* section *Nigri* and ochratoxin A accumulation in grapes [see 36]. Recently, Pandin et al. [37] have suggested that biofilm formation may be important for the establishment and subsequent dispersion of bacterial biocontrol agents, and to facilitate overcoming environmental stress. This may indeed be an important consideration in the formulation and application of bacterial BCAs, for both plant disease and mycotoxin control.

Ecophysiological approaches may be a way to improve the quality of inocula, especially of propagules for more effective control of mycotoxins [38, 39]. While sorghum grain has been effectively used for atoxigenic *A. flavus* strain production and delivery, the optimum conditions for production of environmentally stress tolerant inocula have not often been examined. Mohale [8] showed that the production of conidia of the atoxigenic *A. flavus* strain on sorghum grain varied significantly depending on the moisture content (= a_w) of the solid substrate. The maximum production yield was at 0.96 a_w instead of 0.98 (wet) or 0.92 a_w (dry) conditions. Interestingly, while there were slight differences in germination rates under stressed conditions, germ tube extension of the conidia from the 0.92 a_w treatment was best [8]. This suggests that physiological differences in spore quality occurred when produced on sorghum at different a_w levels. The question is whether this would translate into better mycotoxin control. However, there is significant scope for enhancing the quality of formulations to obtain better mycotoxin control. Previous work with formulations of *Pichia anomala* for control of ochratoxin A in stored wheat grain colonised by *P. verrucosum* showed that yeast cell quality and formulation affected control [39].

5. Conclusions and future perspectives

The focus should be on BCAs for controlling both growth and mycotoxin as opposed to controlling growth only. Partial control of growth might indeed lead to higher accumulation of mycotoxins and thus reduce the quality of the harvested commodity, especially cereals and nuts. Because the growing season of many crops is long and the environmental conditions are under constant fluctuation, more research is needed to link the life cycle of mycotoxigenic pathogens and BCAs in order to find the best application time and dose ratios at different key stages of the growing season. Perhaps these improvements will facilitate higher adoption by farmers that will see the benefit of reducing the number of chemical fungicide applications. This could be part of a more holistic integrated management strategy where application of BCAs is combined with fewer fungicides sprays. Indeed integrated pest management (IPM) could be envisaged by combining BCAs for mycotoxin control with entomogenous fungi for pest control and a minimum of pesticide/fungicide inputs, especially in sustainable production systems. Pests are a major cause of damage to ripening crops, and facilitate the entry of mycotoxigenic fungi. Thus, combinations of BCAs based on entomogenous fungi in combination with those able to control toxigenic fungi could possibly provide a synergistic impact on levels of toxin control achieved.

Furthermore, a better understanding of the effects that sub-lethal doses of chemical fungicides might have on the BCAs would also need to be better understood. This combined with the environmental conditions could have an effect on the BCA capacity to reduce toxin production. Recent studies with antifungal protein from specific species (e.g. *Penicillium chrysogenum*) have suggested that improved mycotoxin control can be achieved by combining such compounds as part of an integrated strategy [40].

For the future, a key aspect which has not been addressed is whether climate change (CC) scenarios will influence the efficacy of BCAs to control key mycotoxigenic fungi and whether formulations available now will be as effective as under present prevailing conditions. Recent studies with entomogenous fungi suggest that efficacy to control pests may be compromised under such environmental stresses [41]. This may require a re-evaluation of formulation technologies to ensure resilience and that efficacy can be maintained under such changed conditions. This may be important in the context of studies which suggest that under CC conditions some mycotoxigenic fungi may be stimulated to produce increased mycotoxin levels. Overall, for sustainable crop protection strategies, an integrated approach for using BCAs for control of toxigenic fungi and relevant pests with reduced chemical inputs would be beneficial.

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Table 1. Conidial spore yields of atoxigenic *A.flavus* strain (AFL2-) on sorghum grain under different water activity levels, and the mean spore germination and germ tube length of conidia from these treatments when grown on Tap Water Agar modified to 0.96 a_w with polyethylene glycol 200, after incubation at 25°C for 24 h (from Mohale, 2013).

a_w[1]	Spore yield (log_{10}/g sorghum)[2]	Spore germination (%) ±SD[3]	Germ tube length (µm) ±SD
0.98	8.8[a]	90.67±1.6[a]	43.62±13.5[a]
0.96	9.2[b]	97.67±2.3[b]	50.55±11.8[a]
0.92	8.5[a]	98.67±4.1[b]	116.62±28.7[b]

[1] Water acitivity of sorghum grain. [2]Values are means of three replicates. [3]Means in a column followed by the same letters are not significantly different (p>0.05).

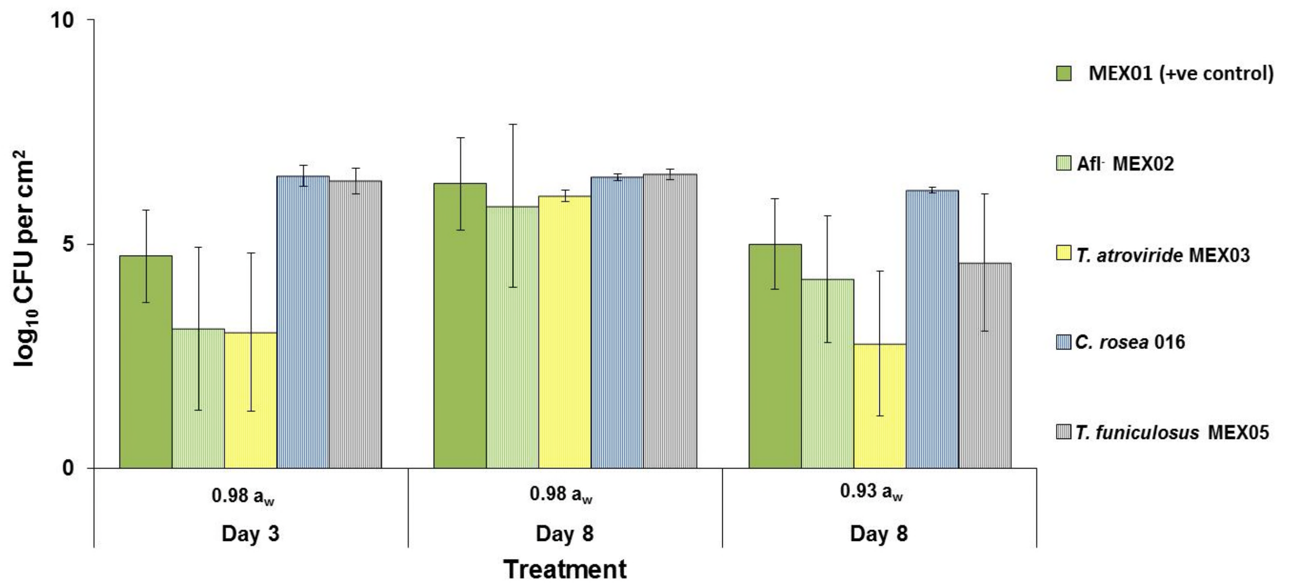

Figure 1. Sporulation of the toxigenic *A. flavus* strain (MEX01) (Log_{10} conidia/cm^2) during co-inoculation with different potential BCAs on senescent maize leaves at 0.98 (-2.8 MPa water potential) and 0.93 a_w (-9.8 MPa water potential) at 30˚C, harvested after 3 and 8 days. Bars indicate SEM. There were no significant difference ($p < 0.05$) between treatments (Tukey's Honest Significant Difference.

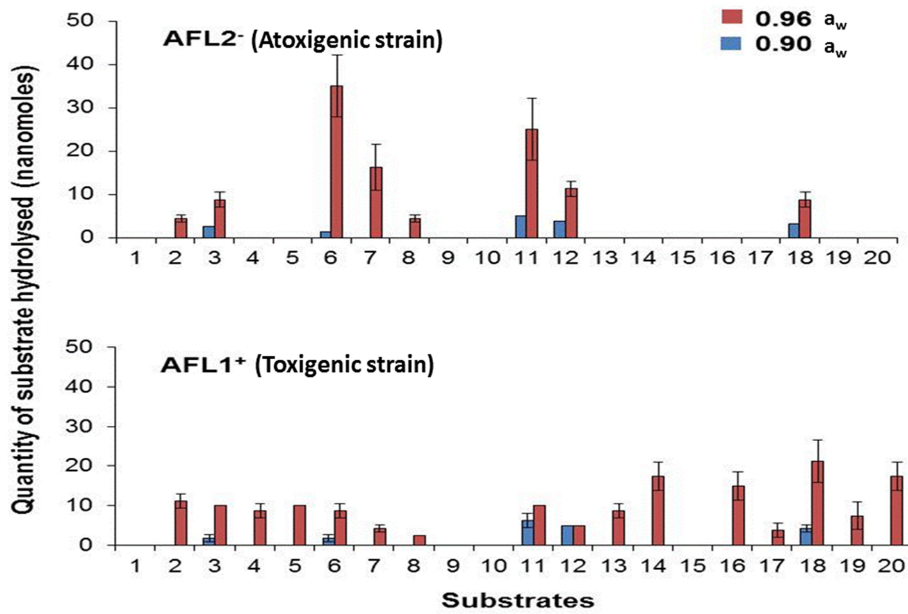

Figure 2. Comparison of production of 19 hydrolytic enzymes by atoxigenic and toxigenic strain of *A. flavus* at 0.96 and 0.90 a_w [from 8]. Key to enzymes assayed: 1, Control; 2, alkaline phosphatase; 3, Esterase (C4); 4, Esterase lipase (C8); 5, Lipase (C14); 6, Leucine arylamidase; 7, Valine arylamidase; 8, Cystine arylamidase; 9, Trypsin; 10, α-chymotrypsin; 11, Acid phosphatase; 12, Naphthol-AS-B1-phosphohydrolase; 13, α-galactosidase; 14, β-galactosidase; 15, β-glucuronidase; 16, α-glucosidase; 17, β-glucosidase; 18, N-acetyl-β-glucosaminidase; 19, α-mannosidase; 20, α-fucosidase.

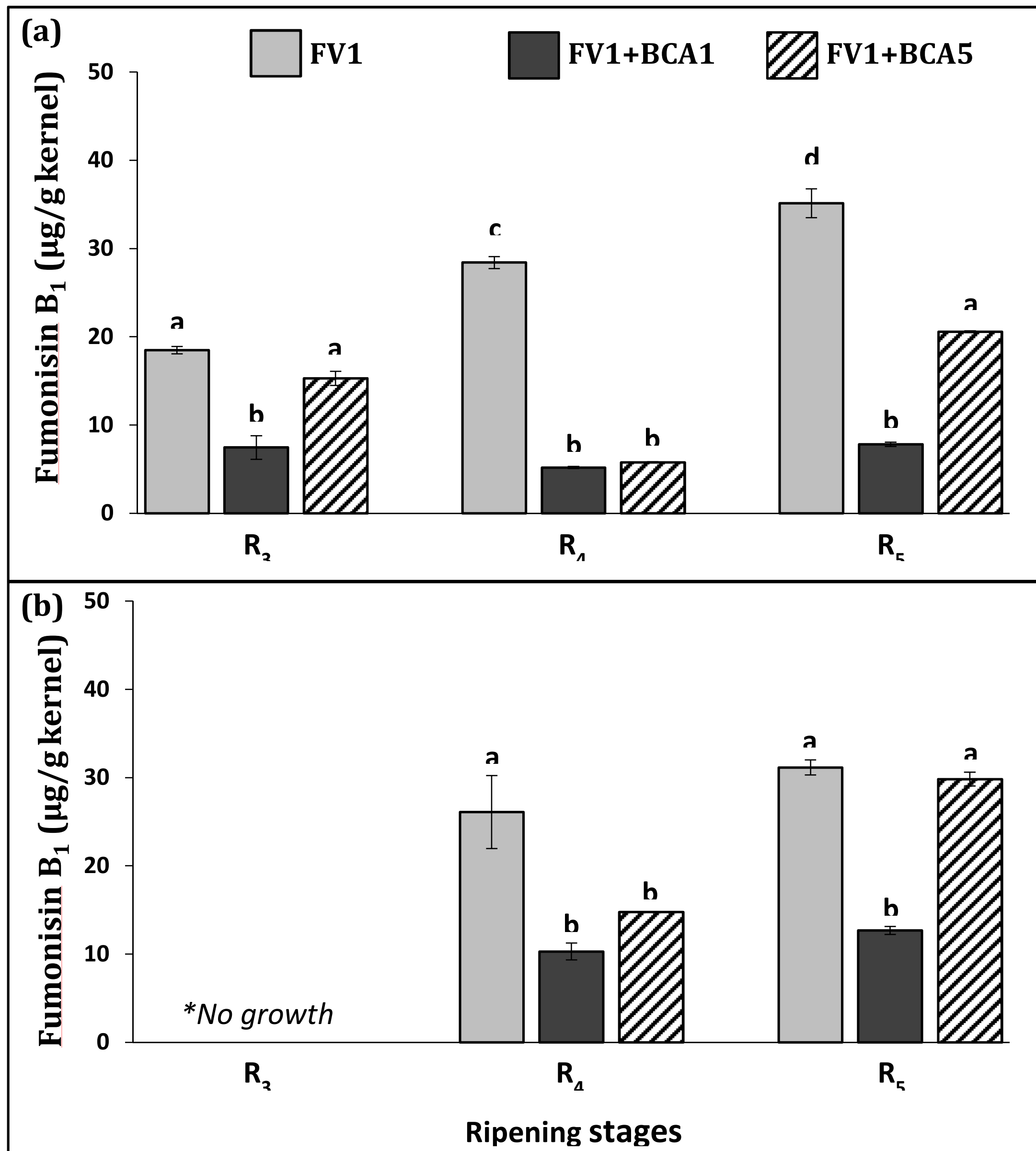

Figure 3. Fumonisin B_1 production by FV1, FV1+BCA1 (gram positive bacterium) and FV1+BCA5 (*C.rosea* 016) on maize cobs of different ripening stages (R3, R4, R5) after 10 days incubation at (a) 25°C and (b) 30°C. Data are means of triplicates. Bars are SEs. Different letters indicate significant difference ($p \leq 0.05$) using Fisher's Least Significant Difference (LSD). Adapted from [9].