Efficacy Assessment of Inhibitory Fungal Concoctions Against Mycotoxin Fungi Affecting Maize Grain Quality in Western Kenya

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Abstract

Limited biological control products exist for use against mycotoxins in maize fields of Kenya's smallholders. In response, this study aimed to assess the capacity of four fungal isolates to suppress the proliferation of mycotoxin fungi and disruption of fungal diversity in field conditions. Field assessments were done in Kibos and Sega sites of Western Kenya during the long and short rain seasons of 2020 while post-harvest assessments were done at University of Eldoret Crop Protection Laboratory. The experiment was laid in a split-plot arrangement in RCBD with four replications. The subplots consisted two susceptible varieties to aflatoxins (Duma and Punda milia) while the sub plots consisted seven treatments including *Trichoderma harzianum, Monascus* species*,* fungal isolate MCMT3, fungal isolate MCBT4b, co-inoculation treatment (*Monascus* sp*,* MCMT3, and MCBT4B), Aflasafe KE01[TM] (positive check) and control (negative check). Inoculations of the isolates were done at 7days after silk emergence at 4ml per ear, while Aflasafe KE01[TM] was applied two weeks before flowering. At post-harvest 5 grains per subplot were cultured in PDA in triplicates and incubated at 25-27°C aseptically. Data on percentage incidences of AF, OTA, PAT and PCN fungi, and number of diverse fungi was collected and subjected to descriptive statistics using Microsoft Excel Spreadsheet Software of Microsoft 365 version. *Monascus* spp. and *T. harzianum* concoctions best suppressed mycotoxin fungal incidences but did not differ significantly with MCMT3, MCMT4b, and the co-inoculation of isolates MCMT3, MCMT4b, and *Monascus* spp. Co-inoculation of *Monascus* spp., MCMT3, and MCMT4b displayed no synergism in suppressing mycotoxin fungi. In conclusion, *Monascus* spp., *T. harzianum*, MCMT3 and MCMT4bhave an inhibitory capacity against *A. flavus, A. parasiticus, A. nomius*, *A. niger P. corrylophilum,* and *P. auratiogriseum* in field conditions. Isolates MCMT3, MCMT4b and *Monascus* species are recommended to control mycotoxins in maize and other crops susceptible to toxigenic fungal infections.

Keywords: efficacy, biocontrol agents, mycotoxins, *Zea mays*

1. Introduction

Maize (*Zea mays*) is an important staple crop for billions of people globally (Kaushal *et al.,* 2022). In Kenya, it is a preferred crop and it is produced by 80% of farmers for self-consumption, sale, or both (Gachara *et al.,* 2022). The crop is also valued for its nutritional properties and contributes to 68% cereal of daily intakes per head/capita, 35% energy, 32% protein, and 50% daily calorie intake in Kenya (Schroeder, *et al*., 2013). The total land area in Kenya under maize is estimated as 1.5 million hectares (KALRO, 2022). However, average countrywide yield stands at 1.8 tons/hectare which is notably below the potential of 6 tons per hectare (Njeru *et al*., 2022). These production amounts are highly insufficient to meet the current per capita consumption demands of 100kg (Gacheri*et al*., 2022).

Mycotoxins exacerbate maize yield losses in Kenya through food contamination (Nji *et al*., 2022). The most profiled mycotoxins in Kenyan maize include aflatoxins, ochratoxins, fumonisin, patulin, penicillic acid and zearalenone (Kagot *et al.,* 2022; Wafula *et al.,* 2022). Acute and chronic mycotoxicosis exposures result to slow and quick onset of symptoms leading to hospitalization or death (Kagot *et al.,* 2022). Mycotoxicosis incidences have been reported in Kenya since 1981; for example, in 2004, aflatoxin poisoning resulted in over 100 human deaths (Brown*et al.*, 2013; Mahuku, *et al*., 2018).The risk remains pertinent as 75% of Kenyan maize is produced by resource-poor farmers under conditions that could predispose the crop to mycotoxin contamination(Kang'ethe, *et al*., 2017). Unfortunately, the sequential processes from production to consumption of the crop lacks sufficient mechanisms to ensure food safety.

Towards sustainability, biological control strategies have been focused primarily on mitigating aflatoxins in maize. The commonly used strategy is the utilization of non-aflatoxin (atoxigenic) strains to limit aflatoxin contamination on crops (Dorner, 2004; Mamo *et al*., 2022). This gives atoxigenic strains a competitive edge by excluding their aflatoxin-producing relatives, decreasing the potential for contamination in crops and the environment (Ortega-Beltran& Bandyopadhyay, 2021; Zhang *et al*., 2020). The formulation and registration of

AflaSafe KE01[TM]for the control of aflatoxins in Eastern Kenya is a success story for this strategy (Cotty& Mellon, 2006; Okun *et al*., 2015). However, this is insufficient since AflaSafe the only registered biological product in Kenya for the control of aflatoxins and its use is limited to Eastern Kenya. The alarm is more strident as no biological control products exist to mitigate the accumulation of other major mycotoxins in field conditions.

In filling this gap, this study sought to develop a new product (concoction) composed of locally sourced atoxigenic fungi against aflatoxin (AF), Ochratoxin-A (OTA), patulin (PAT), and penicillic acid (PCN)-producing fungal species. The goal was to aid in developing early intervention systems against mycotoxins in the field, especially in maize production zones of Western Kenya. The first step involved isolating and characterizing all fungi from grain (maize and groundnut) and soil samples from Western Kenya. *In-vitro* assays were then done to determine non-toxin-producing fungi that can suppress the growth of toxin-producing fungi and eventually reduce the overall potential amount of mycotoxins produced. This study used the best performing antagonists from the *in vitro* assays to formulate efficacious biological concoctions for field evaluation.

2. Materials and Methods

2.1 Site Characteristics
The field experiments were laid in Kibos and Sega sites located in the Western region of Kenya in the long rain and short seasons of 2020. Kibos site is located in Kisumu County, 1184m above sea level, latitude -0.06994N, longitude 34.81688 E, soil pH between 5.0 and 6.0, sub-humid with 1464 mm average rainfall/annum, 23°C average temperatures/day (Juma, Musyimi, & Opande, 2018). Sega site is located in Siaya County1120m above sea level at latitude 0.250425995 N and longitude 34.20243912 E. Ferralsols and Acrisols are the dominant soil types in this area. The site receives annual rainfall of between 1170 and 1450 mm and temperature ranges of 15 to 30°C (Okaron, 2017; Owino *et al*., 2015).

2.2 Planting and Experimental Design
The experiment was laid in a split-plot arrangement in Randomized Complete Block Design (RCBD) and replicated four times in two sites. The Main Plots were two maize varieties susceptible to aflatoxin fungi, that is, Duma and Punda milia. The Sub Plots consisted of seven treatments including *Trichoderma harzianum, Monascus* species*,* fungal isolate MCMT3, fungal isolate MCBT4b, co-inoculation treatment (*Monascus* sp*,* MCMT3, and MCBT4B), AflaSafe KE01[TM] (positive check) and control (negative check). Plot dimensions were 3.75m by 5m comprising 5 rows and 21 hills with 75cm and 25cm between rows and hills respectively. Nitrogen and Phosphorus in a Di-Ammonium Phosphate fertilizer (DAP) (18% N, 46% P and 2.5% S) fertilizer were applied at equivalents of 22.5kg and 25.13kg per hectare respectively. Nitrogen and phosphorus rates per $18.75m^2$ plot were 0.042 kg and 0.047kg respectively. The first and second weeding activities were done 3 weeks and 8 weeks after planting. At top dressing, 65g of N per plot (250g CAN per plot and 125kg/ha) was applied immediately after the first weeding. First season planting was done during the long rains of March-May in 2020 while the second season done during the short rains of October-December in 2020.

2.3 Inoculum Preparation and Treatment Application
Conidia from the plates were washed by pouring 10ml distilled water on the surface of the pure culture and gently scrubbing it with a sterile glass rod for maximum extraction of spores. The suspension was decanted into clean conical flasks, and a solution of four suspensions per litre was made. For co-inoculation treatments, individual inoculums were mixed at a rate of 1:1. The inoculum was further preserved by refrigeration at 4°C. Inoculation of the suspension inoculant made from successful antagonists was done 1 week (7 days) after the emergence of silks from between 50-70% of the plants. Ears were inoculated with 4ml of the inoculum using a 5ml capacity syringe. AflaSafe KE01[TM] was applied two weeks before flowering (7th leaf stages) at a 40 Kg/ha rate following the manufacturer's specifications and as described by Atehnkeng, *et al*., (2008).

2.4 Kernel Assessments for Fungal Diversity
At physiological maturity, 20 ears per subplot were harvested, where one portion of grain was carefully dried and stored in dry conditions. Randomly five grains were sampled near the opening of the ears, where mycotoxin fungi were more likely to infect the seeds. The maize grains were surface sterilized using 1% sodium hypochlorite for 60 seconds and then rinsed thrice using distilled water. Commercial TM Media Potato-dextrose agar (PDA) was prepared as described in the previous experiment. Streptomycin antibiotic was mixed with the PDA media to prevent bacterial contamination (Gulbis *et al*., 2016). The grains were then inoculated into sterile Petri dishes containing sterile PDA media and incubated in aseptic conditions of 25-27°C. Fungi growing from the incubated seeds were identified directly using mycelial colour, mycelial texture, and the morphological features of the conidia and conidiophores (Gulbis *et al*., 2016). Three replicates of five incubated seeds per plate were assessed to determine the incidences of AF-producing fungi (*A. flavus, A. parasiticus* and *A. nomius*), PAT and PCN-producing fungi (*P. corrylophilum,* and *P. auratiogriseum*) and OTA-producing fungi (*A. niger*). The number of

fungal species per plate was also determined to assess the extent of fungal diversity per plate.

2.5 Statistical Analysis

Data on the performance of single and co-inoculations of the bio-control agents, standard commercial product, and the control experiment were subjected to descriptive statistics using Microsoft Excel Spreadsheet Software of Microsoft 365 version. Line and bar graphs with error bars were drawn to depict trends with respect to site and maize variety during the long rain and short rain seasons of Western Kenya.

3. Results

3.1 Effect of bio-control agents on incidences of mycotoxin fungi

During the long rains season *Monascus* species, MCMT3, MCMT4b, *T. harzianum* and the co-inoculation treatment did not differ significantly in percentage incidences of AF, PAT, PCN, and OTA-producing fungi in both sites. *Monascus* species, MCMT3, MCMT4b and the co-inoculation treatment recorded less than 15% incidences of mycotoxin fungi. These levels differed significantly from the control experiment in both sites except for those of the co-inoculation treatment in suppression of PAT & PCN-producing fungal incidences in Sega site (Figure1).
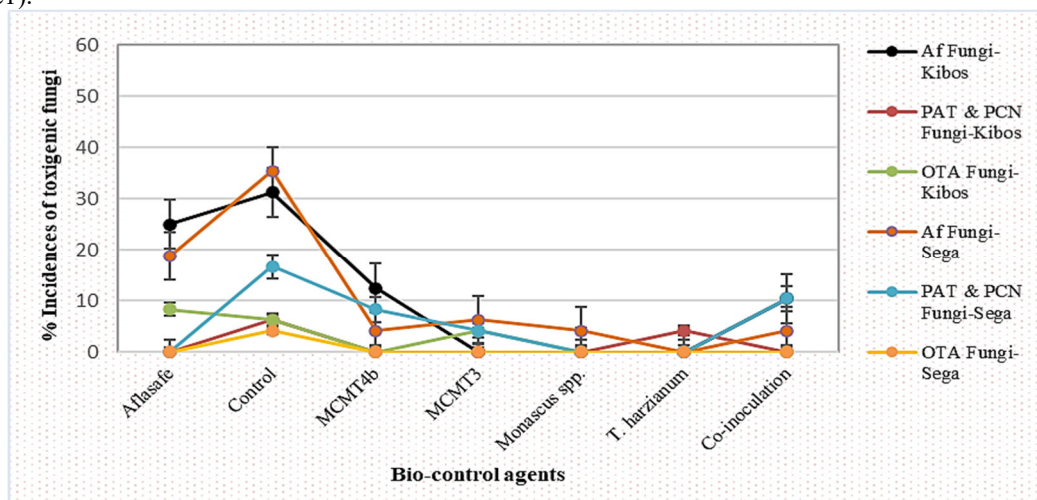

Figure 1. Effect of bio control agents on percentage incidences of mycotoxin fungi during the long rains

Conversely, during the short rains season *Monascus* species, MCMT3, MCMT4b, *T. harzianum* and the co-inoculation treatment did not depict notable differences in their effect on percentage incidences of AF, OTA, PAT and PCN producing fungal species in Sega site. Similar trends were observed in the Kibos site except for isolates MCMT4b and MCMT3, which did not differ significantly with AflaSafe KE01[TM] on their effect on AF, PAT and PCN-producing fungal species (Figure 2). Isolate MCTM3did not differ significantly with the control in percentage incidences of AF, PAT, PCN and OTA producing fungi in Kibos site only. Similar observations were made on isolate MCMT3 against AF and OTA fungi in Kibos site (Figure 2).
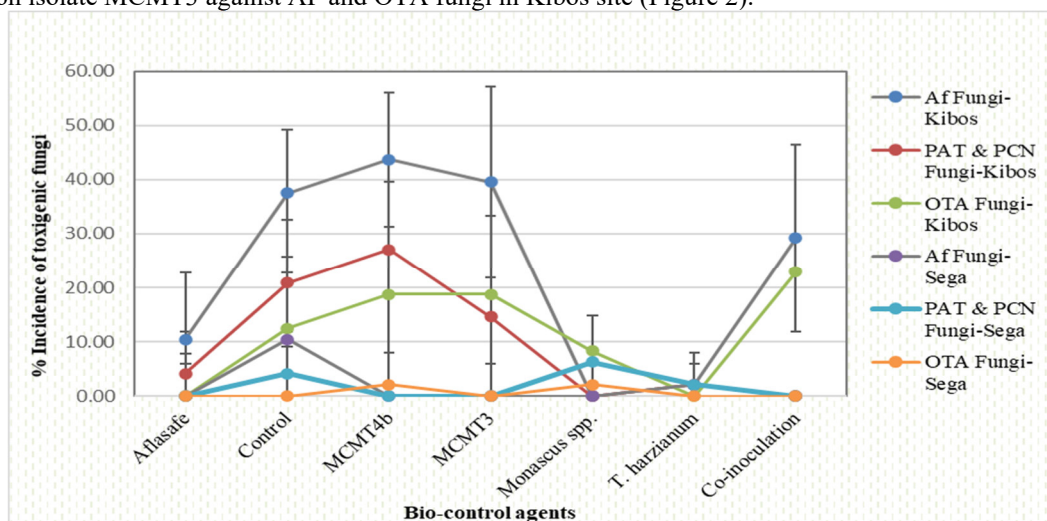

Figure 2. Effect of bio-control agents on percentage incidences of mycotoxin fungi during the short rains season

3.2 Effect of bio-control agents on fungal diversity

Regarding fungal diversity, *Monascus* species*, MCMT3, MCMT4b, T. harzianum* and the co-inoculation treatment did not differ significantly on their effect on fungal diversity during the long and short rains in Kibos site. Similar trends were observed in Sega site except for *Monascus* species and *T. harzianum* which recorded the least diversity and differed significantly with isolate MCMT3 and the co-inoculation treatment in the long rains season (Figure 3).
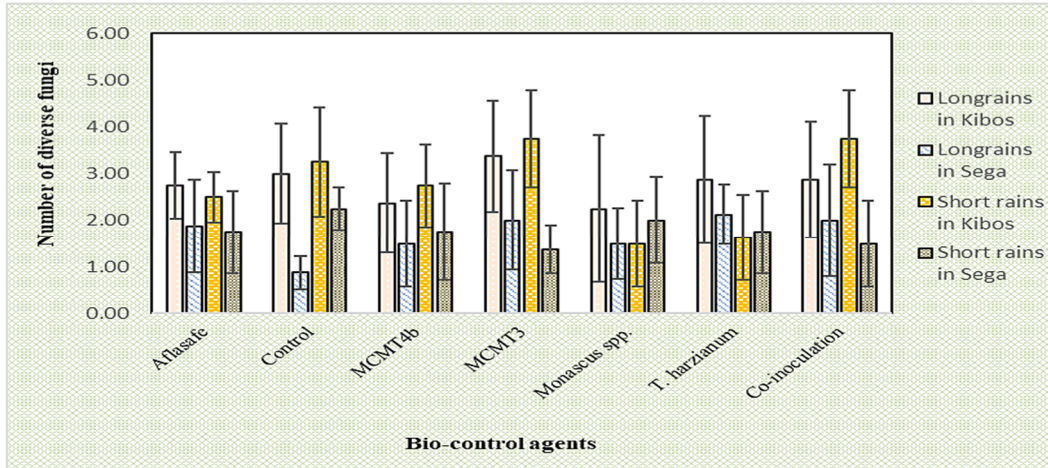

Figure 3. Effect of bio-control agents on fungal diversity in the long rains and short rains seasons

3.3 Effect of maize variety on performance of bio-controls

Percentage incidences of AF, OTA, PAT and PCN producing fungi revealed no significant effect of maize variety on the performance of *Monascus* species*, MCMT3, MCMT4b, T. harzianum* and the co-inoculation treatment. However, there were higher incidences of AF fungi in Punda milia variety than in Duma variety (Figure 4). No clear trends on fungal diversity across the two varieties as affected by bio control agents were observed (Figure 5).
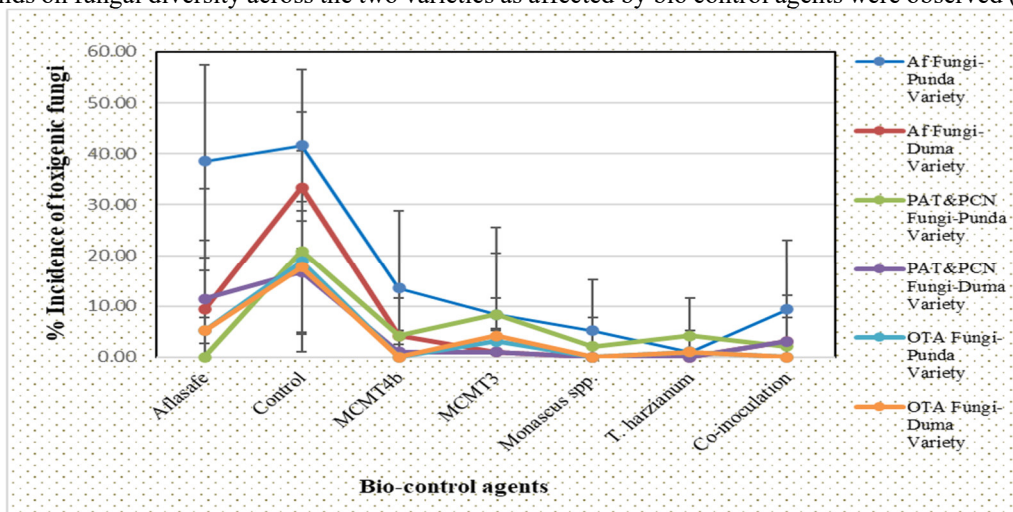

Figure 4. Effect of bio-control agents across maize varieties against incidences of mycotoxin fungi
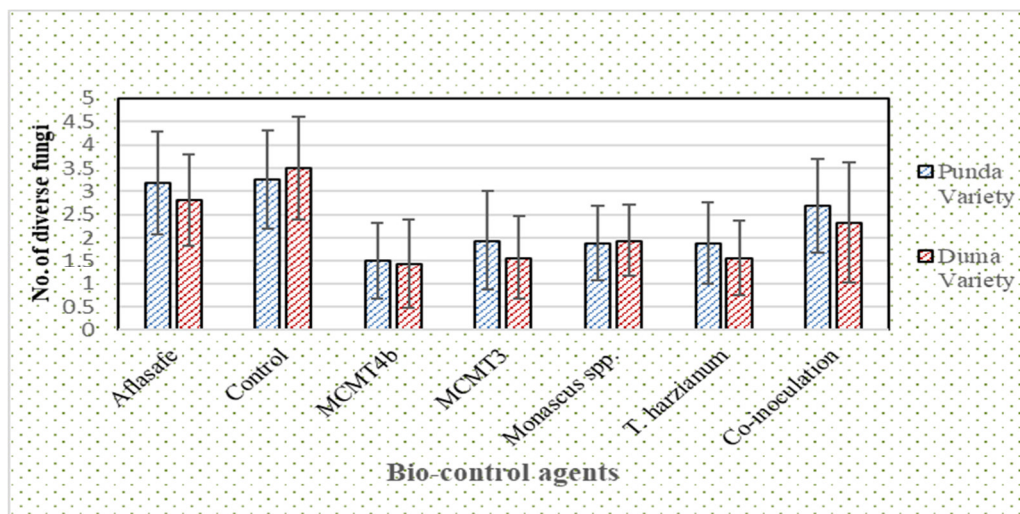
Figure 5. Effect of bio-control agents across maize varieties on fungal diversity

3.4 Effects of bio-control agents on grain quality

Efficacy levels of bio-control agents against mycotoxin fungi were observed in grain quality assessments of harvested ears. Ears treated with *Monascus* species*, MCMT3, MCMT4b, T. harzianum* and the co-inoculation treatment had the best quality kernels in terms of colour. Discolouration effects of mycotoxin fungi were effectual on untreated kernels and AflaSafe KE01[TM] – treated kernels. Infected grains appeared yellowish-green and had traces of mold growth along the longitudinal clefts (Figure 6).

Figure 6. Discolouration effects of mycotoxin fungi on harvested maize kernels

4. Discussion

The post-harvest fungal diversity assessments revealed that *Monascus* species*, MCMT3, MCMT4b, T. harzianum* and the co-inoculation treatmentreduced incidence levels of mycotoxin fungi in field conditions. These variations were also evidenced by the discolouration effects of mycotoxin fungi in harvested kernels. However, general incidences of mycotoxin fungi increased in the short rains season from the long rain season except for aflatoxin fungi for AflaSafe KE01[TM] treatment in the Kibos site of Kisumu County. Krnjaja *et al*., (2019) found that plant density and weather patterns affect *Aspergillus* spp. and *Penicillium* spp incidence levels in maize fields. For this study, the differences in weather patterns and plant densities beyond the experimental area could have resulted in such differences. The results of this study may not ascertain the incidences of aflatoxin-producing fungi in AflaSafe KE01[TM]. This is because the product contains atoxigenic strains that were not distinguished from the toxigenic strains of *Aspergillus* species. It is a crucial study gap, and future studies should consider performing genetic characterization of these species to enhance accuracy.

The effects of maize variety on treatment performance were not distinct. However, Punda milia variety exhibited higher incidences of aflatoxin fungi revealing host preference characteristics of the fungi. Krnjaja *et al*., (2019) had similar results where variations between varieties on susceptibility of toxigenic fungi were not clearly displayed. However, Blandino *et al*. (2017) asserted that maize varieties vary in their severity levels of ear rot. Such variations foster varietal differences in their susceptibility to mycotoxin fungi. Soni *et al*. (2020) found that a 14-kDa trypsin inhibitor is a critical determining factor of susceptibility and resistance. This protein disrupts the normal growth of *A. flavus* by causing spore rapture and anomalous increase of the hyphae (Pechanova & Pechan, 2015; Chen *et al*., 2016).

The pathogenesis-related protein (PR-10) also plays a crucial role in resistance development. The silencing of *pr*-10 genes subject kernels to heat sensitivity which increases the sensitivity of grains to high temperatures and ultimately causes poor transcription of the *pr*-10 genes (Dhakal *et al*., 2017). The development and genetic composition of the rachis are also critical for mycotoxin accumulation. Studies on *A. flavus*, the most abundant mycotoxin, indicate that it uses the rachis as a channel for its spread on the kernels (Pechanova *et al*., 2010). Jeremy and Tibor (2021) had similar results as they found that the rachis suppresses *A. flavus* growth and reproduction. These results infer that maize varietal differences in rachis development are key determinants of varietal performance on *A. flavus* resistance.

Synergism was also not observed since the performance of the co-inoculation (MCMT3, MCMT4b and *Monascus* species) was equal to that of the individual isolates. The inability of the mixed concoction to outperform individual isolates could be due to antagonism between the bio-control agents (BCA). Xu *et al*. (2011) found similar results and concluded that antagonism between bio-control agents is more likely to occur in theoretical and practical contexts than synergism. These isolates were tested in their live forms; hence chances of competitive exclusion and antibiosis were more likely. Combining these isolates to colonize a species is a crucial research gap. Although assessments on fungal diversity on treated kernels revealed insignificant variations, this assessment was not sufficient. Thambugala *et al*., (2020) recommended that determining the non-target effects of bio-control agents and using molecular techniques to identify and track BCAs' pathways is crucial. It will help determine the best approach to leverage their antifungal properties in managing mycotoxins.

5. Conclusion

Monascus species*, MCMT3, MCMT4b, *T. harzianum* and the co-inoculation (*Monascus* species*, MCMT3 and MCMT4b) treatmentsignificantly reduced incidences *A. flavus, A. parasiticus, A. nomius, A. niger, P. corrylophilum,* and *P. auratiogriseum* in field conditions. Single and co-inoculations of isolates MCMT3, MCMT4b, and *Monascus* species had no significant differences in reducing *A. flavus, A. nomius, A. parasiticus, A. niger, P. corrylophilum,* and *P. auratiogriseum* incidences in field conditions. Punda milia and Duma varieties had no significant influence on the performance of the tested biological concoctions.

Isolates MCMT3, MCMT4b and *Monascus* species are recommended to control mycotoxins in maize and other crops susceptible to toxigenic fungal infections.

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