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In-vitro Inhibitory Indices of Selected Fungal Isolates against Mycotoxin Fungi

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Abstract

Limited fungal-based biocontrol products are available for use against mycotoxins in food and feed industry in Kenya. In filling this gap, i*n-vitro* inhibitory assessment of six mycotoxin and nine non-mycotoxin species isolated from Western Kenya were placed on growth media using dual and modified plating techniques to determine the percentage inhibitions, capacity to form inhibition zones and degree of general antagonism on growth of mycotoxin fungi. The cultures were incubated at 25-27^{o}C under 12-hour dark and 12-hour light conditions aseptically. Observations were made 10 days after incubation. Fungal isolates tested for their antagonistic effect on mycotoxin fungi were MCMT4b, MCMT3, MCHB2, *T. harzianum, Monascus* species, *Biatrospora* species, *P. endophytica*, *C. olivaceum,* and *Epichloe* species. Mycotoxin fungi tested were *A. flavus, A. parasiticus, A. nomius*, *P. corrylophillum, P. auratiogriseum* and *A. niger*. More than 80% growth inhibitory indices against mycotoxin fungi were expressed by *T. harzianum,* MCMT3, MCMT4b and *Monascus* species. Also, MCMT3, MCMT4b and *Monascus* species formed the largest inhibition zones against mycotoxin fungi. Fungal isolates MCMT3, MCMT4b, *Monascus* species and *T. harzianum* have growth suppression effect against *A. flavus, A. parasiticus, A. niger, P. corrylophillum,* and *P. auratiogriseum in-vitro.* More elaborate identification of the unidentified fungi, genetic characterization and field efficacy assessments of these isolates is recommended.

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Introduction

Food and feed safety is a global challenge due to mycotoxin contamination in warm regions across the globe (Eshelli *et al*., 2018; Truong *et al*., 2022). It is a significant challenge to sustain quality food and feed production, especially in most areas of sub-Saharan Africa (Nleya *et al*., 2018). However, it is nearing a catastrophic level in Kenya, with the country now ranking high in terms of severity and frequency of mycotoxin poisoning, often with human fatality (Kimanya, 2015; Tan, 2020). Mycotoxins are of high importance because they contribute to grain nutritional and quality losses of up to I billion metric tons on world's agricultural produce yearly (Ayofemi Olalekan Adeyeye, 2020). For example, exposure of humans to aflatoxins at even at low levels can cause cancer and several other health complications, but death is often the result of high and acute level exposure (Awuchi *et al*., 2020; Muthomi, 2018). The mycotoxin problem cuts across the agricultural value chain, affecting farmers, traders, markets, and consumers (animals and humans) (Danso *et al*., 2018).

For sustainable management of these toxins around the world, very few approved biological control products are available to manage mycotoxins in grains at preharvest globally. For instance, in 2015, the first fungal biocontrol product, AflaSafe KE01^{TM}, was developed for use in Kenya (Migwi *et al*., 2020). However, prior to efficacy testing of potential bio-control agents against mycotoxin-producing fungi, testing the target and non-target effect of fungal interactions between the toxin producers (toxigenic) and non-producers (atoxigenic) is necessary (Degola *et al*., 2021; Mylroie *et al*., 2016).

For effective development of efficacious biocontrol agent, the abundance and distribution of fungi by their geographical location in three major crop-producing regions of Kenya were classified (Salano, 2015). However, since the aflatoxin problem is still persistent in Kenya, it is essential to identify additional efficacious biocontrol agents (fungi) with broad spectrum activity against a wide range of mycotoxin fungi. Therefore, this study aimed at determining the *in-vitro* inhibitory capacities of selected fungal species against mycotoxin-producing fungal isolates obtained from Western Kenya.

Materials and methods

Study site, isolate selection and incubation

This study was carried at the University of Eldoret, Department of Seed, Crop and Horticultural Sciences in Plant Pathology Laboratory. Six isolates of mycotoxin-producing fungi and nine isolates of mycotoxin non-producers were sourced by sub-culturing the preserved isolates obtained from the previous study done by Mwatabu *et al*. (2022). The isolates were selected by examining their growth rate on potato dextrose agar (PDA) media versus that of mycotoxin-producing fungi such as *Aspergillus flavus.* Commercial TM Media PDA was used as source of nourishment to the fungal isolates. Thirty-nine grams were carefully added on 1 litre of distilled water. Homogeneity of the contents was achieved using a TOS-6048RD Flask orbital laboratory shaker and then autoclaved in an electric vertical autoclave at 121 psi for 15 minutes. Streptomycin antibiotic was added to the sterilized media to prevent bacterial growth.

In-vitro Suppression of Mycotoxin Fungi

Inhibition activity of selected fungi against mycotoxin-producing fungi was tested by growing the test fungi and the antagonists on PDA using two media placement techniques (Maurya, Singh, & Tomer, 2014; Kumar *et al.*, 2020). Selected antagonistic fungi tested were identified as *Biatriospora* species, *C. olivaceum, Epichloe*species, MCHB2 (unidentified), *P. endophytica, T. harzianum,* MCMT4 (unidentified) and *Monascus* species. These were tested against three aflatoxin-producing fungal species (*Aspergillus flavus, Aspergillus parasiticus,* and *Aspergillus nomius*); two patulin and penicillic

acid-producing fungi (*Penicillium corrylophillum* and *Penicillium auratiogriseum*); and one Ochratoxin A-producing fungi (*Aspergillus niger*).

The first in-vitro placement method was dual culture, where 5mm discs of the antagonist and the test fungi were cultured opposite at equidistant points from the periphery of the media within the Petri dish. The second placement technique was a modification by the author where the antagonist fungus was placed at four equidistant points from the periphery/perimeter of the plates and the mycotoxin fungi cultured at the centre (Fig. 1). All cultures containing the combinations were replicated three times.

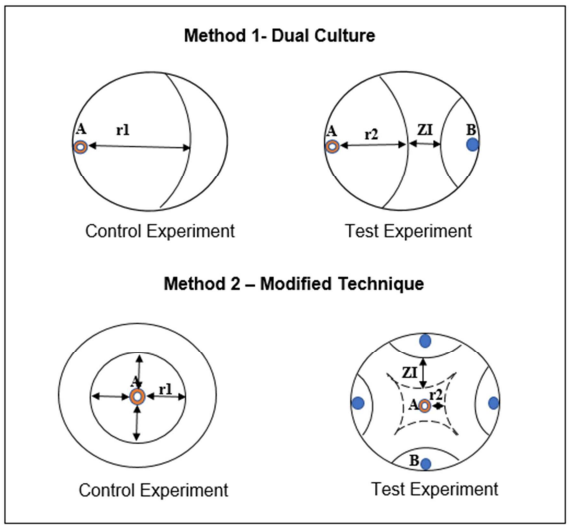

Fig. 1. Diagrammatic illustration of the placements and positioning of the mycotoxin fungi (A) (*Aspergillus* and *Penicillium* species) and the antagonist fungi (B) using methods 1 and 2.

Data Collection

After seven days of culture incubation, data on mycelia growth of the toxigenic fungi and width of the zone of inhibition were collected, and percentage inhibition was calculated according to the formula by Maurya *et al*., (2014).

$$\mathbf{I}(\%) = \frac{r1 - r2}{r1} x\ 100$$

Where: I = Percent inhibition, r_1 = furthest radius (in mm) of the mycotoxin fungi in the control experiment and r_2 = the radius of the mycotoxin fungi adjacent towards the initial position of the antagonistic fungi.

In addition to percent inhibition, other variables measured were the width of the inhibition zone (IZ) in millimeters. The degree of antagonism was also measured using a scale of 1-5 growth rate ranking where 1 = the antagonist grew on the entire plate and/ or completely covered the test fungi, 2 = the antagonist covered over 2/3 of the plate, 3 = the antagonist and the test fungi both covered an equal area of the media surface, 4 = the test fungi grew to over 2/3 of the surface and 5 = the test fungi covered the entire plate (or almost) and overgrew the antagonist (Kucuk & Kyvanc, 2011).

Statistical data analysis

The data on inhibitory indices was subjected to analysis of variance on Genstat statistical software version 16.0, VSN International Ltd at 5% level of significance. The mean differences among the nine treatments consisting of eight biocontrol fungi, a synthetic chemical and a control experiment were separated using Duncan's Multiple Range Test (DMRT). Visual observations were presented in figures to illustrate degree antagonism against mycotoxin fungi.

Results and discussion

Results

Inhibitory index of mycotoxin fungi on dual culture technique

Trichoderma harzianum expressed the highest inhibition (> 60%) under in-vitro conditions in dual culture techniques against most mycotoxin fungi including *A. flavus*, *A. parasiticus*, *A. niger*, *P. corrylophylum* and *P. auratiogriseum*. However, with dual culture technique, *A. nomius* recorded less inhibition to *T. harzianum* isolate but its growth was more controlled by isolate MCMT4b. Other than *A. nomius*, isolate MCMT4b exhibited similar inhibition (>40%) against *P. auratiogriseum*. In contrast to *T. harzianum* and

MCMT4b isolates, *Monascus* species and isolate MCMT3 were effective against all the mycotoxin fungi tested (Fig. 2).

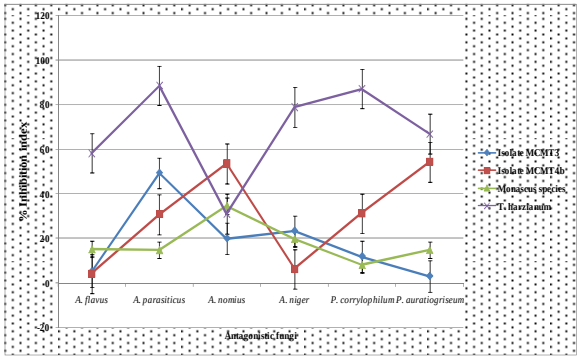

Fig. 2. Percentage inhibition of mycotoxin fungi in-vitro by antagonistic fungal isolates from Western Kenya using the dual (1:1) culture method.

Modification of dual culture showed different and increased inhibition for *T. harzianum* against all mycotoxin fungi with more than 80% inhibitory index. However, just like the dual culture technique, the remaining isolates expressed low (< 40%) inhibition against all mycotoxin fungi under *in vitro* conditions (Fig. 3).

In-vitro inhibition based on the rating scale of 1-5

Based on the inhibition rating scale, *Trichoderma harzianum,* MCMT3, and *Monascus* species rated the best isolates out of the nine. Specifically, *Trichoderma harzianum* was the best-performing antagonist, with a mean of < 2.5 against all mycotoxin-producing fungi. However, this fungal isolate did not differ significantly with *Monascus* species and MCMT3 with respect to inhibition against *P. auratiogriseum*. Also, isolate MCMT4b exhibited antagonism with a mean of less than 3.0 against *A. Parasiticus, P. corrylophylum,* and *P. auratiogriseum* on average. Fungal isolates that did not exhibit inhibition include *Biastrospora* species, *C. olivaceum, Epichloe* species, MCHB2, and *P. endophytica* (Table 1).

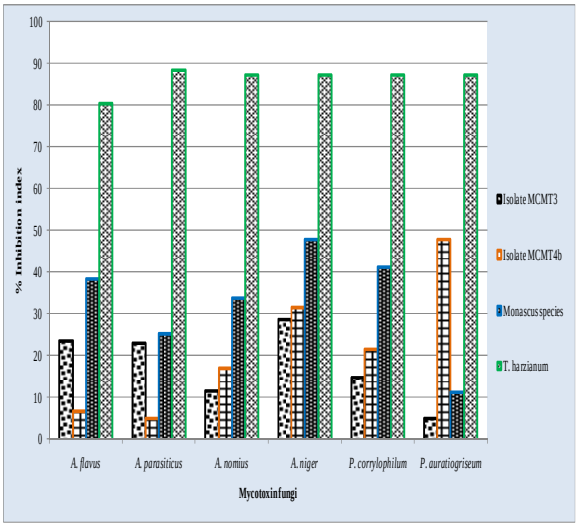

Fig. 3. Percentage inhibition of mycotoxin fungi in-vitro by antagonistic fungal isolates from Western Kenya using the modified method.

Table 1. Inhibition levels of mycotoxin fungi based on inhibition scale (1-5) using the dual culture method.

Antagonist Fungi	*A. flavus*	*A. parasiticus*	*A. nomius*	*A. niger*	*P. corrylophilum*	*P. auratiogriseum*
Biatriospora sp	4.133de	5e	4.6d	5f	4.333e	5e
C. olivaceum	3.7d	4.3d	4.367d	3.867d	3.9d	4.433d
Epichloe sp.	4.333e	4.767e	4.733d	4.8ef	4.6ef	3.933c
Isolate MCMT3	2.5b	2.667b	3.267b	2.667b	3.133c	2.367a
Isolate MCMT4b	3.8d	2.9b	3.367bc	3.433c	2.5b	2.867b
Isolate MCHB2	4.967f	3.967d	4.433d	5f	4.867f	40633de
Monascus sp.	3.133c	3.433c	3.267b	3.867d	3.133c	2.367a
P. endophytica	3.767d	4.7e	4.067cd	4.6e	4.767f	4.6de
T. harzianum	1.367a	1.7a	1.767a	2.133a	1.733a	2.1a
Mean	3.522	3.715	3.763	3.93	3.663	3.589
Probability	<.001	<.001	<.001	<.001	<.001	<.001
S.E	0.1764	0.0339	0.1302	0.0714	0.14	0.1567
S.E.D	0.238	0.1855	0.3408	0.1356	0.0525	0.1868
% CV	5	0.9	3.5	1.8	1.4	4.4

In-invitro inhibition based on formation of inhibition zones

In the dual culture method, antagonistic fungi that successfully formed inhibition zones against the tested mycotoxin fungi were isolates MCMT3, MCMT4b, *T. harzianum, Monascus* species, and *P. endophytica*. However, in the modified method, only isolates MCMT3, MCMT4b and *Monascus*

species formed inhibition zones of more than 1 mm. In terms of specific inhibitory fungus, *Monascus* species formed the widest inhibition zones against *A. flavus, A. parasiticus, A. niger,* and *P. auratiogriseum*. Isolates that did not exhibit inhibition by forming inhibition zones against mycotoxin fungi include *Biastrospora* species, *C. olivaceum, Epichloe* species, and Isolate MCHB2 (Fig. 4).

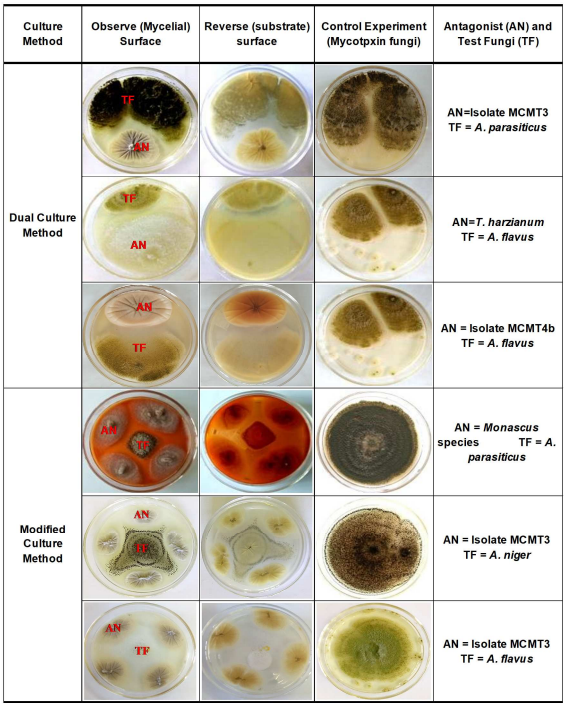

Fig. 4. Inhibitory zone formation from the interaction between antagonistic and mycotoxin fungi under in-vitro conditions on dual and modified methods.

Discussion

The results on *in-vitro* suppression of mycotoxin fungi revealed that Isolate MCMT3, Isolate MCMT4b, and *Monascus* species positively inhibited *A. flavus, A. parasiticus, A. nomius. A. niger, P. corrylophylum* and *P. auratiogriseum*. By forming the best inhibition zones against mycotoxin fungi, these antagonists expressed their capacities to produce inhibitory chemical compounds against fungal growth (Lass-Flori, Perkhofer & Mayr, 2010). The variation in degree of inhibition expressed by the differences in inhibition zones could be due to variations in types and amounts of antifungal compounds produced. It may also be due to variations dominant inhibition mechanisms such as antibiosis, competitive exclusion, mycoparasitism and hyperparasitism (Abdallah *et al*., 2018). However, further studies would be needed for confirmation on the modes used by each of the isolated fungi in Western Kenya.

Among the best antagonistic fungi against mycotoxin fungi, *Monascus* species exhibited unique antagonistic effects by producing red pigments when interacting with mycotoxin fungi *in-vitro* and these observations on pigment production are consistently similar to the findings by Liu *et al*., (2018). The observed red and orange pigments have been explored more extensively in their use in food pigments. Kim *et al*. (2006) found that *Monascus* species pigments with L-and D- forms of amino acids exhibited antimicrobial characteristics against *A. niger* and *P. citrinum*. In addition, *Monascus* species also have a wide application in the pharmaceutical industry (Agboyibor *et al*., 2018). Their application in detoxifying aflatoxins, patulin, penicillic acid and ochratoxin A is underexplored despite the demonstrated potential using specific mycotoxin fungi *in-vitro*.

Similarly, isolates MCMT3 and MCMT4 had similar suppressive effects against mycotoxin fungi. Even though they were unidentified conclusively, they possessed characteristics such as undulate margins, umbonate elevations, and irregular forms surfaces on growth media. These two fungi also had wrinkled surfaces on media but not of equal sizes; their colony colour ranges from pewter to tan, respectively, surrounded by white margins at the observe/front surface. Conidia are enclosed in intact bitunicate asci, a common characteristic in members of the class leutiomycetes of the phylum *Ascomycota* (Ekanayaka *et al*., 2019; Schoch, Lopez-Giralrdez, Sung, & Townsend, 2009). These isolates formed clear inhibition zones against all

the mycotoxin-producing fungi which could be evidence of the possible production of lytic enzymes and secondary antifungal metabolites.

Conclusion

Isolate MCMT3, Isolate MCMT4b, *Monascus* species, and *T. harzianum* have a significant suppressive effect against *A. flavus, A. parasiticus, A. niger, P. corrylophylum,* and *P. auratiogriseum in-vitro.* Also, MCMT3, Isolate MCMT4b, and *Monascus* species form clear inhibition zones in *in-vitro* assays against *A. flavus, A. parasiticus, A. niger, P. corrylophylum,* and *P. auratiogriseum*.

Recommendations

There is need for proper taxonomic identification if MCMT3 and MCMT4b fungi both at morphological and molecular levels. This study also recommends the efficacious assessment of these antagonistic fungi against mycotoxin fungi under field conditions with use of specific host plant such as maize.

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