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Inhibition Studies on Isolated *Aspergillus flavus* Using Solvent Extract from *Trichoderma viride* and *Bacillus subtilis*

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ABSTRACT

Aflatoxins are a group of the most toxic microbial metabolites found in foods and feeds. This study was conducted to obtain solvent extract from *Bacillus subtilis* and *Trichoderma viride* cultures and to assess the level of inhibition against the growth of *Aspergillus flavus* in vitro. Methanol (70%) was used to extract 7-day old (*T. viridae*) and 2-day old (*B. subtilis*) cultures. Filter paper impregnated disc method was used to determine the extract efficacy in inhibiting *A. flavus* growth on potato dextrose agar media using the extracts singly and in combination at varying doses. Analysis of variance was performed on all data with the general linear model. Least significant difference test was performed to compare treatment means at the 5% level. The result demonstrated a significant antagonistic effect of the solvent extracts against the growth of *A. flavus* indicating the presence of bioactive compounds which mitigates *A. flavus* growth at various doses of application. The highest level of inhibition individually was observed in the assay of solvent extract from *B. subtilis* at double dose compared to *T. viride* at double dose. The mixture of *B. subtilis* and *T. viride* solvent extract at double dose demonstrated the highest level of inhibition of *A. flavus* than individual extract at various doses. The relatively high level of inhibition confirms the presence antifungal substances in the solvent extract obtained from *Bacillus subtilis* and *Trichoderma viride* which could be used as bio-fungicides; an ideal alternative to synthetic fungicides in the control of *Aspergillus flavus*.

Keywords:
Aflatoxin,
Aspergillus flavus,
Bacillus subtilis,
Trichoderma viridae,
Methanol

INTRODUCTION

Aspergillus subgenus *circumdati* section *flavi*, has been researched globally for its commercial use and toxigenic ability. The aflatoxigenic species *Aspergillus flavus* L., *Aspergillus parasiticus and Aspergillus nomius*, which cause serious problems globally in agricultural commodities are best known for their colonization of cereals, legumes and tree nuts (Kwanashie, 2019; Shabeer, *et. al*., 2022). The fungus has been also been found to be associated with fruits, such as watermelon and pawpaw (Wata *et al*., 2025). Under favourable conditions of harvesting and storage, agricultural products may be invaded by toxigenic strains of *Aspergillus flavus* and this could result in the accumulation of a highly toxic class of mycotoxins commonly referred to as aflatoxins, which when consumed, are toxic (chronic and/or acute) to mammals. Postharvest rot typically develops during harvest, storage and in transit. *A. flavus* infections can occur while hosts are still on field (pre-harvest), but often show no symptoms until postharvest storage and/or during transportation.

The presence of these fungi does not always indicate that harmful levels of aflatoxin are present, but does indicate a significant risk. *A. flavus* is an opportunistic human and animal pathogen (Munshed and Jameel, 2024). It can cause aspergillosis (an infection caused by *Aspergillus*) as well as produce aflatoxin, a carcinogenic mycotoxin. It has also been reported to cause nasal sinus lesions and other invasive diseases. *Aspergillus flavus* infections will not always reduce crop yields alone; however, post-harvest disease can reduce the total yield by 10 to 30%. Presently, more than 50 countries have established regulations for controlling aflatoxins concentrations for maize, raw and processed groundnuts products (Fabunmi and Kwanashie, 2019). Despite aflatoxin regulatory standards, unpackaged food for domestic consumption is not effectively regulated. This means aflatoxin-contaminated grain can easily enter the Nigerian consumption stream due to low awareness and prevention of *Aspergillus flavus* and its health impacts among consumers and sellers (Oluwakanyinsola *et al*., 2023).

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Management strategies primarily consist of measures designed to principally prevent or reduce the invasion of *Aspergillus flavus*, which includes the use of breeding for resistance and other cultivar characteristics, cultural practices such as planting and harvest dates, tillage practices, crop rotation, plant population, irrigation, sanitation, and crop protection chemicals (insecticides and fungicides) or biological control which refers to a microbial antagonist that keeps the disease-causing agents in check by reducing their populations to economically insignificant levels around the susceptible or target host organ/tissue, resulting in no disease incidence (Gong, 2024). Several bacterial and fungal bio-control agents have already been screened all over the world to identify potential antagonists to *A. flavus*. Researchers have identified a host of potential bio-control agents that work against *A. flavus*, including antagonistic bacteria (*Pseudomonas* spp and *Bacillus subtilis*), fungi (*Trichoderma* spp), and actinomycetes (*Streptomyces* spp) strains (Kumari *et al*., 2017; Ren *et al*., 2022). Under the changing agriculture scenario, the only technology that seems promising to manage the disease without disturbing the equilibrium of harmful and useful composition of environment and ecosystem is the use of more and more biological control agents (Sharma *et al*., 2014).

Trichoderma viridae and *Bacillus subtilis* are bio-control agents that are strong opportunistic invaders, fast growing, prolific reproducers and are also powerful antibiotic producers even under highly competitive environment for space, nutrients, and light (Thakaew and Niamsup, 2013; Ren *et al*., 2022). The mechanisms employed by these organisms to antagonize phytopathogenic fungi include competition, colonization, direct mycoparasitism and antibiosis. The mechanism of antibiosis is commonly reported among many species including microorganisms and plants. This involves the production and secretion of small size diffusible compounds or antibiotics produced by these species which inhibit the growth of other microorganisms (Bharose and Gajera, 2018; Nehra et al., 2021). This study sought to explore the potential of *Trichoderma viridae* and *B. subtilis* in restricting mycelial growth of *A. flavus* in vitro thereby reducing or eliminating the accumulation of aflatoxins in agricultural produce/products.

MATERIALS AND METHODS

Experiment location

The experiment was carried out at the Mycotoxin Laboratory in the department of Crop Protection, Institute of Agricultural Research (IAR) Samaru, Faculty of Agriculture, Ahmadu Bello University, Zaria. The experiment was conducted under an ambient temperature and condition.

Sample collection

Culture samples of *Aspergillus flavus, Bacillus subtilis and Trichoderma viride* were obtained from the Culture room at the Department of Crop Protection, Institute of Agricultural Research (IAR) Samaru, Faculty of Agriculture, Ahmadu Bello University, Zaria.

Confirmation of *Aspergillus flavus* from Culture Sample

Sub-cultures of *A. flavus* were made by transferring mycelia mass from the culture samples using a heat sterilized metal pick to Potato Dextrose Agar amended with streptomycin (PDAs) plates to multiply the masses and obtain pure cultures. The pure cultures were preserved in PDAs slants for further analysis. The plates were arranged on a laboratory table and observed daily for seven days for growth of *A. flavus*. The cultures were then identified on the basis of colony characteristics and conidial morphology.

Confirmation of *Trichoderma Viride* from Culture Sample

The culture samples of *Trichoderma viride* were sub-cultured using a heat sterilized metal pick to transfer mycelia mass from the culture sample into petri dishes filled with Potato Dextrose Agar amended with streptomycin (PDAs) plates and pure samples were preserved in PDAs slants for further analysis. The sub-culture of *T. viride* was allowed to grow after 7 days of maturity and were identified on the basis of colony characteristics and conidial morphology.

Confirmation of *Bacillus subtilis* from Culture Sample

The culture samples of *Bacillus subtilis* were sub-cultured using a heat sterilized inoculation loop to transfer masses of the bacterial colony from the culture sample into petri dishes filled with nutrient agar (NA). The pure cultures were allowed to grow for 3 days before harvesting. The colonies of pure bacterial cultures were characterised based on morphological characteristics like colour, size, margin, form, elevation and texture (Varghese and Joy, 2010).

Samples Preparation (Extraction of Metabolites)

The bioactive compounds were extracted by harvesting full grown T*richoderma viride* (after 7 days) and *Bacillus subtilis* (after 24hrs) colonies into Methanol (organic solvent).

The worktable and other apparatus were sanitized thoroughly with 70% Ethanol. Four petri dish containing matured pure culture of *Trichoderma viride* and *Bacillus subtilis* each, were scrapped using a heat sterilized spatula into 15ml beakers of Methanol each kept within ice bags. The culture broths were refrigerated at 4℃ for 24hrs and shaken intermittently using vortex shaker to break frozen metabolites into the organic solvent. The culture broths were then subjected to centrifugation at 4000rpm for 30mins at 4℃. Further separation was done by filtration using Whatman filter paper to separate filtrate from culture supernatant. The cell-free culture filtrates were then stored at 4℃ to be used for further analysis.

Inhibitory Activity Test

The methods described by Horváth *et al*., (2016) was used. The bacteria and fungi isolated from the culture samples had conclusively been identified as *Bacillus subtilis, Aspergillus flavus, and Trichoderma viride.* The culture filtrates of *Bacillus subtilis* and *Trichoderma viride* were used to assay the antifungal activity against the test phytopathogen, *Aspergillus flavus* on filter disc paper of 1.5cm in diameter. Potato dextrose agar was prepared and poured into 36 plates of 9cm petri dishes and the following treatments were laid out.

Inhibitory effects of culture filtrates from *Trichoderma viride* against *Aspergillus subtilis (in vitro*)

Control 1: *Aspergillus flavus* alone

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 7 days.

Control 2: Organic solvent (Methanol) against *Aspergillus flavus*

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 24 hours before 30µl of methanol was added using a pipette on two filter paper discs placed at opposite ends of (PDAs) plates daily for 7 days.

Treatment 1A: Single dose of *Trichoderma viride* culture filtrate against *Aspergillus flavus*

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 24 hours before 30µl of T. viride culture filtrate was added using a pipette on two filter paper discs placed at opposite ends of (PDAs) plates daily for 7 days.

Treatment 2A: Double dose of *Trichoderma viride* culture filtrate against *Aspergillus flavus*

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 24 hours before 60µl of T. viride culture filtrate was added using a pipette on two filter paper discs placed at opposite ends of (PDAs) plates daily for 7 days.

Treatment 3: Single dose mixture of *Trichoderma viride* and *Bacillus subtilis* culture filtrate against *Aspergillus flavus*

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 24 hours before culture filtrate of *T. viride* and *B. subtilis* were mixed together and 30µl of the culture filtrate was added using a pipette on two filter paper discs placed at opposite ends of (PDAs) plates daily for 7 days.

Treatment 4: Double dose mixture of *Trichoderma viride* and *Bacillus subtilis* culture filtrate against *Aspergillus flavus*

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 24 hours before culture filtrate of *T. viride* and *B. subtilis* were mixed together and 0µl of the culture filtrate was added using a pipette on two filter paper discs placed at opposite ends of (PDAs) plates daily for 7 days.

The experiment was laid out in a completely randomised design with three replications. Records of *A. flavus* colony growth (Area of colony covered) was taken daily for 7 days when the culture in the control had covered the plates.

Inhibitory effects of culture filtrates from *Bacillus subtilis* against *Aspergillus subtilis* (in vitro)

Control 1: *Aspergillus flavus* alone

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 7 days.

Control 2: Organic solvent (Methanol) against *Aspergillus flavus*

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 24 hours before 30µl of methanol was added using a pipette on two filter paper discs placed at opposite ends of (PDAs) plates daily for 7 days.

Treatment 1B: Single dose of *Bacillus subtilis* culture filtrate against *Aspergillus flavus*

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 24 hours before 30µl of *B. subtilis* culture filtrate was added using a pipette on two filter paper discs placed at opposite ends of (PDAs) plates daily for 7 days.

Treatment 2B: Double dose of *Bacillus subtilis* culture filtrate against *Aspergillus flavus*

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 24 hours before 60µl of *B. subtilis* culture filtrate was added using a pipette on two filter paper discs placed at opposite ends of (PDAs) plates daily for 7 days.

Treatment 3: Single dose mixture of *Bacillus subtilis* and *Trichoderma viride* culture filtrate against *Aspergillus flavus*

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 24 hours before culture filtrate of *T. viride* and *B. subtilis* were mixed together and 30µl of the culture filtrate was added using a pipette on two filter paper discs placed at opposite ends of (PDAs) plates daily for 7 days.

Treatment 4: Double dose mixture of *Bacillus subtilis* and *Trichoderma viride* culture filtrate against *Aspergillus flavus*

Aspergillus flavus was inoculated at the centre of the plate using a sterilized metal plate pick with streptomycin (PDAs) plates and incubated at 30℃ for 24 hours before culture filtrate of *T. viride* and *B. subtilis* were mixed together and 60µl of the culture filtrate was added using a pipette on two filter paper discs placed at opposite ends of (PDAs) plates daily for 7 days

The experiment was laid out in a completely randomised design with three replications. Records of *A. flavus* colony growth (Area of colony covered) was taken daily for 7 days when the culture in the control had covered the plates.

Data Analysis

The data was analysed in R (version 4.1.0, R core Team). Analysis of variance (ANOVA) was performed on all data with the general linear model (GLM). Least significant difference (LSD) test was performed to compare treatment means at the 5% level.

RESULTS AND DISCUSSION

Confirmation of Culture Samples

Confirmation of *Aspergillus flavus* culture samples

Plate 1 shows cultures of *A. flavus* which were isolated and identified from collected samples. Isolates present with the characteristic dark green colony colouration with the reverse side hyaline (plate 1). Microscopic examination revealed the globose, ellipsoid and slightly spherical conidia.

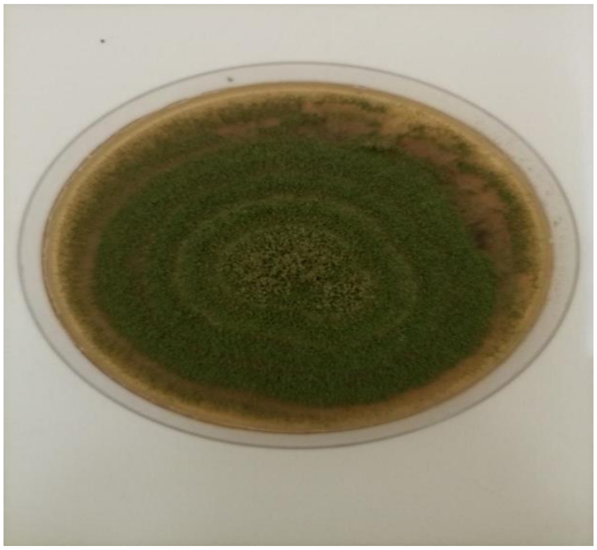

Plate 1: *A. flavus* growing on PDAs showing the characteristics dark green colony colouration.

Aspergillus flavus was isolated and identified. The confirmatory features conform to those described by Diba *et al.*, (2007) on pure cultures colony. Pure cultures of *Aspergillus flavus* identified from macroscopic characterization showed, upon microscopic examination, the characteristic globose, ellipsoid and slightly spherical conidia.

Conformation of *Bacillus subtilis*

The culture samples of *Bacillus subtilis* were isolated and had identical results for the test conducted. Colonies had a mix of round to irregular shaped forms. Colonies were fast growing measuring between 2-5mm in diameter after 48 hours incubation. The margins of colonies were undulating, appearing multiple depressions and crests as a wave. The elevation of colonies appeared relatively flat with a slight gentle protrusion at the centre (umbonate). Colonies were white to creamy in colour and appeared dry and moderately rough (plate IV).

Isolates had motile and rod-shaped cells when observed under the microscope. Gram staining produced the violet coloration indicative of a positive reaction (thick peptidoglycan cell wall). Isolates were found to have the catalase enzyme (a critical enzyme for identification of gram-positive bacteria) but lacked cytochrome oxidase assays. Isolates were negative for Indole (tryptophan metabolism) and Urease (urea metabolism) tests. The methyl -red (MR) test in which the pH of bacterial isolates was measured in a phosphate buffered solution supplemented with peptone and glucose was neutral (MR negative). Isolates were, however, positive for citrate (citrate permease enzyme in the catabolism of citrate as a source of carbon) and Voges-Proskauer (acetoin detection in the catabolism of glucose) tests.

Plate 2: *B. subtilis* growing on PDAs showing the characteristic creamy colony colouration.

Confirmation of *Trichoderma viride*

Identification was made by macroscopic and microscopic analysis of subcultures prepared on diagnostic media. This isolate was identified as *Trichoderma viride*. Morphology of colonies and sporulating material matched species descriptions provided in published taxonomic keys.

Plate 3: *T. viride* growing on PDAs showing the characteristics dark greenish colony colouration

Inhibitory studies against *Aspergillus flavus*

Aspergillus flavus against single dose of *Bacillus subtilis* and *Trichoderma viride*

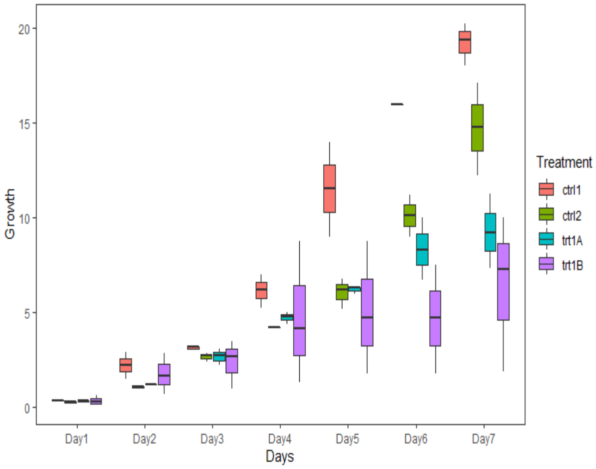

Figure 4.1 Inhibitory effect of *A. flavus* against single dose of *B. subtilis* and *T. viride*

NB:
CONTROL 1: *Aspergillus flavus* colony
CONTROL 2: *Aspergillus flavus* colony against organic solvent (methanol)
TREATMENT 1A: *Aspergillus flavus* colony against single dose of *T. viride*
TREATMENT 1B: *Aspergillus flavus* colony against single dose of *B. subtilis*

INTEPRETATION

Single dose (30µl) of solvent extract obtained from *B. subtilis* and *T.viride* was assessed for its inhibitory effect on the growth of *A. flavus* in vitro (Figure 4.1). The plot below shows that treatments with *Bacillus subtilis* extract, single dose inoculated with *A. flavus* (treatment 1B) showed the lowest A. flavus growth after 7 days. This was followed by treatment 1A (*A. flavus* innoculated against T. viride extract) after 7 days, with control 1 (inoculated *A.flavus* Alone) having highest growth of the fungus than control 2 (*A. flavus* inoculated against Organic solvent, Methanol).

At day 1, treatment 1A and 1B had a non-significant ($p>0.001$) effect on the growth of *A. flavus* compared with control 1 and 2. By day 2 treatment 1A gave significantly low growth of *A. flavus* than treatment 1B compared with the control. Generally, from day 3 to day 7, the growth of *A. flavus* was significantly higher in the control 1 and control 2 when compared with all other treatments. Treatment 1B consistently gave significantly lower growth of *A. flavus* ($P>0.001$) than treatment 1A compared to control 1 and 2. Plate 4 shows *Aspergillus flavus* 24hrs after innoculation with *Bacillus subtilis* and *Trichoderma viride.*

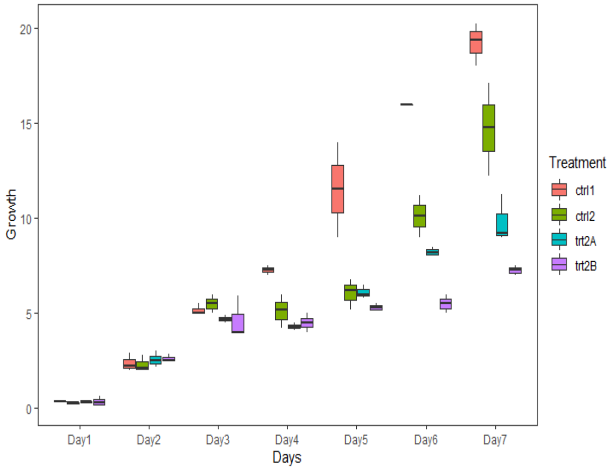

Figure 4.2 Inhibitory effect of *A. flavus* against double dose of *B. subtilis* and *T. viride*

NB:

CONTROL 1: *Aspergillus flavus* colony

CONTROL 2: *Aspergillus flavus* colony against organic solvent (methanol)

TREATMENT 2A: *Aspergillus flavus* colony against double dose of *T. viride*

TREATMENT 2B: *Aspergillus flavus* colony against double dose of *B. subtilis*

INTERPRETATION

Figure 4.2 shows the result of the inhibitory effect of double dose (60µl) of solvent extract obtained from *B. subtilis* and *T. viride* on the growth of *A. flavus* in vitro. The plot below shows that treatments with *Bacillus subtilis* extract, double dose inoculated with *A. flavus* (treatment 2B) showed the lowest *A. flavus* growth after 7 days. This was followed by treatment 2A (*A. flavus* inoculated against double dose of *T. viride* extract) after 7 days, with control 1 (inoculated *A. flavus* Alone) having highest growth of the fungus than control 2 (*A. flavus* inoculated against Organic solvent, Methanol).

Both treatments significantly inhibited the growth of A. flavus when compared with the control. At day 1 and day 2, both treatments show significant difference from the controls in the mitigation of A. flavus growth. By day 2 treatment 2A and 2B gave a significantly($P>0.001$) low growth of *A. flavus* compared with the control. Generally, from day 3 to day 7, the growth of *A. flavus* was significantly higher in the control 1 and control 2 when compared with the treatments. Treatment 2B consistently gave a significantly lower growth of *A. flavus* ($P>0.001$) than treatment 2A compared to control 1 and 2.

Aspergillus flavus **growth against single and double dose mixture of** ***Trichoderma viride*** **and** ***Bacillus subtilis*** **solvent extract**

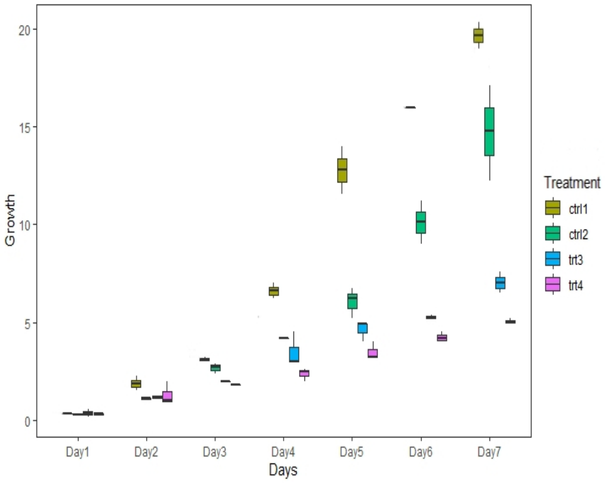

Figure 4.3 Inhibitory effect of *A. flavus* with single and double dose mixture of *B. subtilis* and *T. viride* solvent extract

NB;

CONTROL 1: *Aspergillus flavus* colony

CONTROL 2: *Aspergillus flavus* colony against organic solvent (methanol)

TREATMENT 3: *Aspergillus flavus* colony against mixture of *T. viride* and *B. subtilis* single dose.

TREATMENT 4: *Aspergillus flavus* colony against mixture of *T. viride* and *B. subtilis* double dose.

INTERPRETATION

Figure 4.3 shows result of the inhibitory effect of solvent extract obtained from the mixture *B. subtilis* and *T. viride* solvent extract at single and double doses of the extract on the growth of *A. flavus* in vitro. The plot below shows that treatments with the mixture of *Bacillus subtilis* and *T. viride* extract at double doses inoculated with *A. flavus* (treatment 4) showed the lowest *A. flavus* growth after 7 days. This was followed by treatment 3 (*A. flavus* inoculated against single dose of the mixture) after 7 days, with control 1 (Inoculated *A. flavus* Alone) having highest growth of the fungus than control 2 (*A. flavus* inoculated against Organic solvent, Methanol).

Both treatments significantly inhibited the growth of *A. flavus* when compared with the control. At day 1 and day 2, both treatments show significant difference from the controls in the mitigation of *A. flavus* growth. By day 2 treatment 3 and 4 gave significantly ($P>0.001$) low growth of *A. flavus* compared with the control. Generally, from day 3 to day 7, the growth of *A. flavus* was significantly higher in the control 1 and control 2 when compared with the treatments. Treatment 4 consistently gave significantly lower growth of *A. flavus* ($P>0.001$) than treatment 3 compared to control 1 and 2.

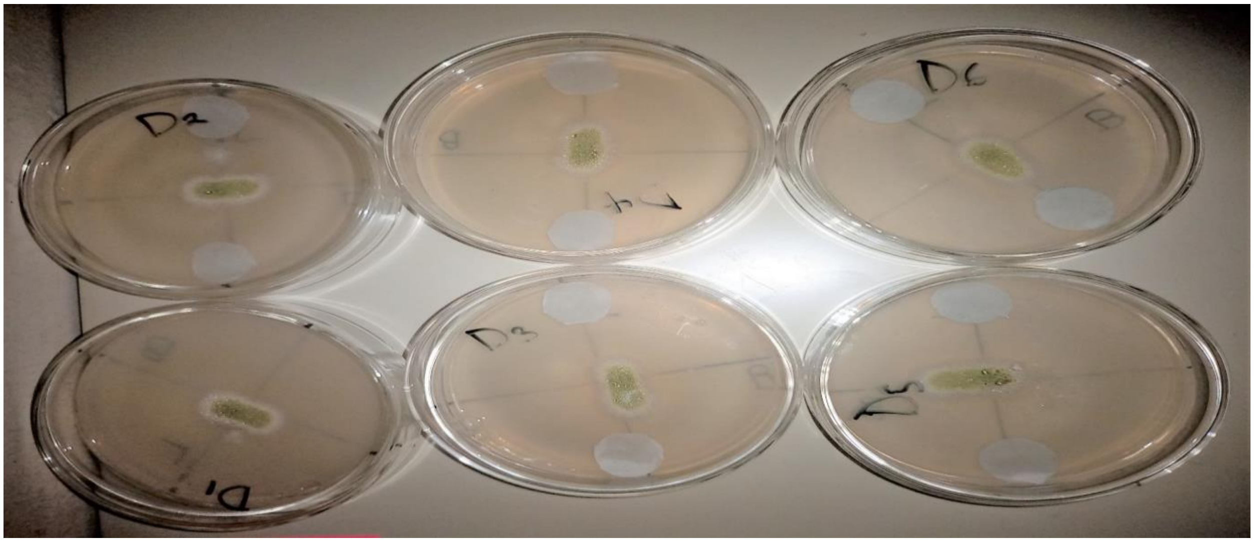

Plate 4: *Aspergillus flavus* growth 48 hours after application *Bacillus subtilis* extract and *Trichoderma viride* at different doses and mixture on potato dextrose agar

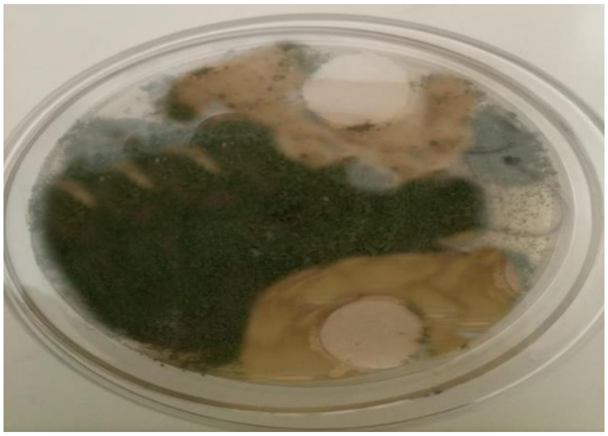

Plate 5: *Aspergillus flavus* growth after 7 days against solvent extract obtained from *Bacillus subtilis* and *Trichoderma viride*

Researchers have serially isolated microorganisms from natural environments that have proven ability to restrict fungi growth, particularly the toxigenic *A. flavus*, including strains of *Bacillus* and *Trichoderma* (Yuan et al., 2023, Yue et al., 2022). Taghavi et al. (2025) reported that *Bacillus subtilis*, *Bacillus licheniformis* and Bacillus *axarquiensis* isolated from soil samples exhibited potent antifungal activity against multiple fungal species, spotlighting the potential of this genus as an effective biocontrol option. Similarly, Ma et al. (2025) isolated three strains from maize, soybean, and rice seeds that effectively inhibited *A. flavus*, which were identified as *B. velezensis* and *B. amyloliquefaciens*.

In this present study, the methanolic extract from *B. subtilis* and *T. viride* was assayed for antagonistic activity against *A. flavus* growth. The assay was conducted in vitro and at single and double doses of individual solvent extract and mixture of both solvent extracts. Observations showed that both extracts at all level of doses and mixture successfully and significantly impaired the growth of *Aspergillus flavus* mycelia i.e., colony growth. The extract from *B. subtilis* at single and double doses was shown to restrict the growth of *Aspergillus flavus* than extract from *T. viride* compared to the control over a period of 7 days. The application of solvent extract at double dose (60µl) of *Bacillus subtilis* and *Trichoderma viride* showed more antagonistic effect against the growth of *Aspergillus flavus* than single dose (30µl) of solvent extract applied. The assay of *Aspergillus flavu*s with mixture of solvent extract of *Bacillus subtilis* and *Trichoderma viride* at single and double doses showed the most antagonistic effect on the growth of *A. flavus* than other individual treatments at both doses.

A number of studies have described the combined application of viable cells (including spores) of *Bacillus* and *Trichoderma*. Rigobelo et al. (2024) reported that co-inoculating soybeans with these microorganisms enhanced plant growth by modifying the soil microbial community. Another soyabean study reported that while the combination did not change plant fresh or dry mass, it significantly down regulated the spore density of pathogenic microorganisms, including *Aspergillus* species (Braga et al. 2024).

Results from this study suggest that metabolites present in the methanol filtrates of both microorganisms are responsible for their inhibition activity. Thus, we infer that the inhibitory zones observed between *Bacillus* and *Trichoderma* against *A. flavus* likely arise from certain metabolites produced during culturing that interfere with mycelial growth of *A.*

flavus. The production of antibiotics by *B. subtilis* and their application in the management of plant pathogens have been variedly reviewed. Kadhim and Matloob (2025) reported a lytic factor in the cell wall and Nareendra-Babu *et al.,* (2015) described catalytic enzymes such as chitinase, protease, cellulase and glucanase and metabolites such as subtilin, bacitracin, subtenolin, bacilonycin and bacillin as antibiotics sequestered by *B. subtilis* which crack and deform the hyphae of fungi, which leads to altered cell structure and function due to the formation of vacuoles within and adjacent cells and protoplast leakage. The production of volatile compounds by isolates of *Trichoderma viridae* tested in vitro against phytopathogenic fungi. These metabolites may include harzianic acid, alamethicins, tricholin, peptaibols, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid (Vey *et al.,* 2001; Ruangwong et al., 2021). *Bacillus subtilis* and *Trichoderma viride* which extracts were used in this study may possess the ability to synthesize some of these active chemical metabolites which, may account for the observed inhibition of *A. flavus* growth in vitro. Quite unlike previous studies that focused on single-species metabolites (Zhao et al., 2023, Dini et al., 2022), we combined the methanol culture filtrates of *B. subtilis* and *T. viridae* to enhance the suppression of *A. flavus* growth and AFB_1 production. This approach was designed to circumvent the mutual antagonism observed between the live strains and thereby amplify the overall inhibitory effect on *A. flavus*.

CONCLUSION

There was the presence of antifungal substances in the solvent extract obtained from *Bacillus subtilis* and *Trichoderma viride* which are effective against effective against the growth of *Aspergillus flavus.* The results showed a marked area of inhibition of *A. flavus* growth, in vitro, especially when incubated with extract from *B. subtilis* at various doses. However, it was also observed that mixture of solvent extract from *B. subtilis* and *T. viride* significantly inhibited the growth of *A. flavus* in vitro in both doses than the individual treatments which confirms the synergistic antagonistic effect of the mixture against *A. flavus* growth.

Therefore, the high level of significance of these solvent extract urges and encourage the need for the adoption and use of bio-fungicides in mitigating the growth of *Aspergillus flavus* and reduce impact of Aflatoxin presence in crop produce, other than synthetic fungicides which are not eco-friendly and are hazardous to the Human health.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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