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# Integration of Biochar with Atoxigenic Strains of *A. flavus* Can Effectively Control Aflatoxin Contamination in Groundnuts (*Arachis hypogaea* L.)

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## ABSTRACT

*An In Vitro study conducted at the NM-AIST Laboratory in Arusha, Tanzania, investigated the efficacy of Biochar in managing aflatoxin contamination in groundnuts, both independently and in combination with Aflasafe. Various concentrations (2 X $10^2$, 2 X $10^4$, 2 X $10^6$, and 2 X $10^8$) of atoxigenic biocontrol strains extracted from AflasafeTZ01 were introduced to the groundnut grains treated with 5%, 10%, 15%, and 20% maize cob Biochar. All groundnuts were inoculated with atoxigenic A. flavus (2 x $10^6$) and incubated at 30°C for seven days before aflatoxin quantification using HPLC. The Analysis of Variance (ANOVA) conducted on the data revealed a significant ($P < 0.001$) difference among the means in reducing aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$. Notably, there existed an inverse relationship between the concentration of Biochar and aflatoxin content, with the most substantial reduction observed at a 10% Biochar rate.*

*Moreover, integrating Biochar with the Aflasafe biocontrol option further decreased aflatoxin contamination. For example, applying 10% Biochar along with 2 x $10^6$ Aflasafe resulted in an impressive 99.99% reduction compared to the control. In comparison, using Biochar or Aflasafe alone led to reductions of 80.6% and 90%, respectively.*

*These findings hold significant implications for the agricultural sector, suggesting that the strategic utilization of Biochar and Aflasafe can substantially mitigate aflatoxin contamination in groundnuts. This offers a promising solution to a significant food safety issue, providing hope for a safer and healthier food supply.*

**Key Words:** Aflatoxin, Aspergillus flavus, Aflasafe, Biochar, Groundnuts, In Vitro.

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## 1. INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is a leguminous crop that originated in the New World and is notably widespread across Mesoamerica and South America (Daudi *et al*., 2018). Archaeological evidence, such as groundnut remnant pericarp tissue found in Peru, suggests its intentional agricultural use dating back approximately 3900-3750 years ago (Mfaume *et al*., 2019). The domestication of groundnuts is evident in archaeological findings from 300 to 2500 BC, primarily in the valleys of the Paraguay and Prarana rivers within the Chaco region of South America(Munsanda *et al*., 2018). In Africa, groundnuts were introduced from Brazil by the Portuguese during the 16$^{th}$ century, as noted by Daudi *et al*. (2018) [1].

In 1946, the crop was introduced in Tanganyika to produce vegetable oil (Katundu et al., 2014). Ever since, it has been the most significant crop for smallholder farmers in Tanzania, providing food, feed, and income for households (Mfaume *et al.*, 2019). The crop is grown in different types of soils, preferably those with more than fifty per cent sand with pH ranges between 4.8 to 7.0 and rainfall range of 600 to 1500 mm per annum (Daudi *et al.*, 2018). Nutritionally, groundnut is rich in fat, protein, carbohydrates, vitamins, and minerals (Abady *et al.*, 2019). However, the crop mostly succumb to aflatoxin contamination at the pre-harvest stage due to its anatomical structure (Kuhumba *et al.*, 2018). Aflatoxin contamination in groundnuts is increasing tremendously regardless of the interventions made due to the crop's and soil's inherent nature as the sole media for both crop and aflatoxin-causing inoculum. According

to the European Rapid Alert System for Food and Feed (2020) database, aflatoxin was the most common mycotoxin in groundnuts among other mycotoxins (Pickova *et al.*, 2021). FAO (2003) asserts that 25% of the world's food products (maize and groundnuts) were significantly affected by aflatoxins. In Africa, the annual monetary loss due to aflatoxin-contaminated groundnut in 2019 was reported to be over $250 million (Mfaume *et al.*, 2019). It is also reported that the annual economic impact caused by the aflatoxin effect on humans in Tanzania was approximately $1,100 (Mfaume *et al.*, 2019). In 2016, Tanzania reported 65 hospitalized patients and 19 deaths in high groundnut-producing districts ( Chemba and Kondoa in Dodoma region) due to aflatoxin (Massomo, 2020).

Currently, primary strategies for managing aflatoxin include implementing good agronomic practices (GAP), biological control, timely planting and harvesting, proper post-harvest handling, effective storage techniques, and chemical control (Ortega-beltran & Bandyopadhyay, 2021). Nevertheless, the combined performance of these techniques has been reported as ineffective in significantly reducing aflatoxin contamination (Abbas *et al*., 2011; Gasperini *et al*., 2019; Maxwell *et al*., 2021; Moore, 2021) requiring studies to explore integrated and effective methods for managing aflatoxin contamination. The literature extensively discusses the use of Biochar for soil improvement and pathogen control, elucidating its effectiveness across various mycopathosystems. Studies indicate that Biochar demonstrates notable efficacy in managing soil-borne pathogens, with reported suppression rates of 86% for fungi, 100% for oomycetes, 100% for viruses, 96% for bacteria, and 50% for nematodes (Iacomino *et al*., 2022). Focusing on fungal soil-borne pathogens, Biochar impact has been observed in species such as *F. oxysporum* f. spp., *Verticillium dahliae*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Macrophomina phaseolina*, *Sclerotium cepivorum*, and *Sclerotium rolfsii* (Akanmu *et al*., 2020; Medeiros *et al*., 2021; Frenkel *et al*., 2017; Rogovska *et al*., 2017; and Xiang *et al*., 2022). The effectiveness of Biochar is influenced by various factors such as the raw materials used, soil type, soil quality, and pyrolysis temperature (Sobczak *et al*., 2020). Reported mechanisms for efficacy of Biochar include the induction of systemic resistance, enhancement of microbial community dynamics in the rhizosphere, elevation of soil pH, and adsorption of phytotoxic compounds originating from plants or microbes (Akanmu *et al*., 2020; Munsanda *et al*., 2018; Tomczyk *et al*., 2020). Furthermore, Biochar could serve as a carrier for delivering both nutrients and microbial inoculants to agricultural soils (Kamali *et al*., 2022). Its unique physical and chemical properties support the growth and activities of beneficial microbes, particularly by preventing desiccation during dry periods (Hale *et al*., 2014; Kamali *et al*., 2022; Luigi *et al*., 2022; Xiang *et al*., 2022). These distinctive properties could be leveraged by integrating Biochar with beneficial atoxigenic *Aspergillus flavus(A. flavus)* to enhance their effectiveness as biocontrol agents. While atoxigenic *A. flavus* biocontrol has been widely endorsed for aflatoxin control in recent years (Abbas *et al*., 2011; Agbetiameh *et al*., 2020; Gasperini *et al*., 2019; Maxwell *et al*., 2021; Moral *et al*., n.d.), its effectiveness may vary depending on site-specific conditions, farming practices and other environmental factors. Therefore, integrating Biochar, which complements and enhances various microenvironments at the preharvest crop stage, is desirable.

It is proposed that combining Biochar with atoxigenic *A. flavus* biocontrol strains (Aflasafe) could enhance their effectiveness in managing Aflatoxin contamination among smallholder farmers in Tanzania. Nonetheless, proven scientific information on this integration is still scanty (Kalus *et al*., 2020). Therefore, the present study aims to evaluate the impact of Biochar on the Aflasafe biocontrol option in mitigating Aflatoxin contamination in groundnuts.

## 2. MATERIALS AND METHODS

### 2.1 Study location

A study was conducted in March of the year 2022 at the Nelson Mandela African Institution of Science and Technology (NM-AIST) Laboratory in Arusha, Tanzania. The NM-AIST is located at latitude 3.40°14′20″ N, longitude 36.79°58′20″ E and altitude of 1199 m.a.s.l. The area has a temperature that ranges between 10 and 30 °C and an average annual rainfall of 1,180 millimetres. The humidity varies between 65 in dry to 90% during cool weather and main rain seasons.

### 2.2 Materials

The toxigenic *A. flavus* was collected from Mikocheni Mycology Laboratory in Dar es Salaam, Tanzania. Aflasafe was bought from agro shops in Arusha, Tanzania. Maize cobs were collected from farmers around NM-AIST to



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prepare Biochar due to the necessity of using locally available materials. Susceptible groundnut seeds that were free from aflatoxin (Red Mwitunde variety), which was obtained from TARI Naliendele, Tanzania, were used in this study.

### 2.3 Methods

#### 2.3.1 Biochar production and characterization

**(i) Biochar production**

The collected maize cob samples were carried to the NM-AIST laboratory for pyrolysis. Pyrolysis was done using a macro furnace at the standard effective temperature of 500℃ for one hour, cooled by natural conversion, pulverized using a heavy-duty grinder, and sieved using a 2mm sieve.

**(ii) Biochar characterization**

Microscopic analysis of biochar was carried out in Motlatsi Phari Institution-South Africa on 2nd December 2023 at the magnification of 200xx and Electrical heating temperature of 500 kV. A research microscope, Nikon Eclipse E-200, with fluorescence attachment, was used to identify Biochar morphological characteristics using a Scanning Electron Microscope (SEM) (Fig 2). Biochar's porosity and pore size were scrutinized using Brunauer-Emmett-Teller (BET). Characterization was done to understand the physical morphology for determination of the ability of materials to absorb solvents.

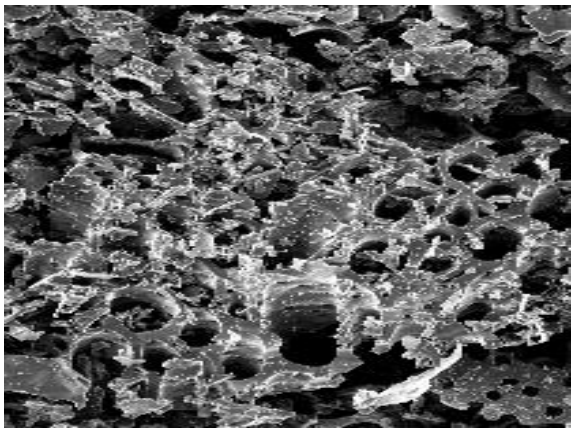

**Figure 1. SEM image showing micro and macro pores in maize cob Biochar pyrolyzed at the temperature of 5000C at NM-AIST macro furnace taken by Motlatsi Phari Institution( south Africa) on June 2023.**

Chemical characterization of Biochar was done at the TARI-Uyole laboratory in Mbeya, Tanzania.

**Note:** B=Boron, Ca=Calcium, CEC=Cation Exchange Capacity, K=Potassium, Mn=Manganese, P=Phosphorus, Si=Silicon, TN=Total nitrogen. The y axis is the concentration of parameters shown in the X axis.

#### 2.3.2 Inoculum preparation and experimental layout

**(i) Inoculum preparation**

The inoculum of the S-type virulent strain of *A. flavus* isolates No. TGS 55-6 was cultured in the 90 mm Petri dishes containing half-strength Potato Dextrose Agar (PDA). The incubation was done at 30°C for seven days to allow the formation of infective spores. The inoculum was then harvested from its culture using distilled autoclaved water, and twin 20 was added for dispersion. The concentration was adjusted to $2.5\times10^6$ spores/mL by using a hemocytometer.



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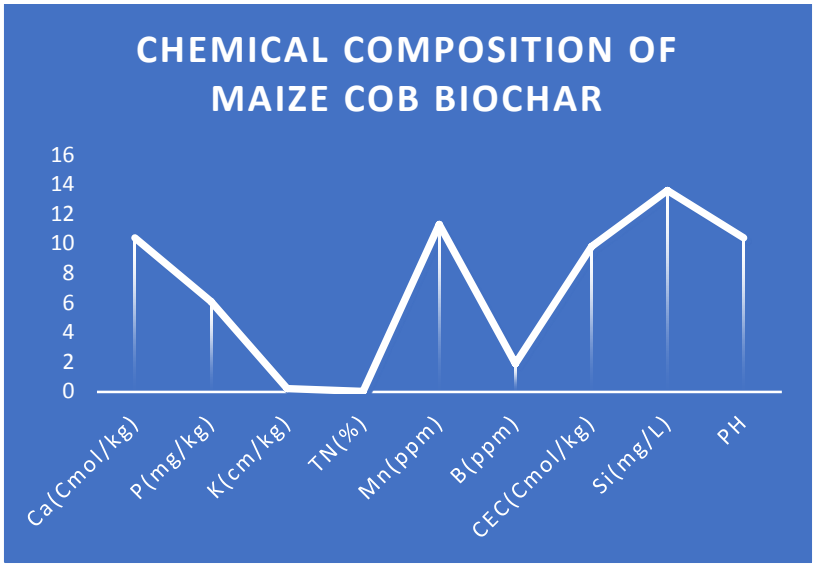


**Figure 2. Chemical composition of maize cob Biochar collected from the farmers around NM-AIST pyrolyzed at 500$^0$C**

### (ii) Experimental layout

The experiment was conducted in a completely randomized design **(**Table 1). Each treatment was replicated three times, and each treatment was observed two times per replicate. The treatments were Biochar, Aflasafe and a combination (Biochar and Aflasafe) at different rates and concentrations.

**Table 1. In vitro Experimental layout at NM-AST laboratory, Arusha, Tanzania, done during the year 2022**

| Treatments | Levels | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2 | F | F | E | E | G | G | H | H |
| 3 | K | K | J | J | I | I | L | L |
| 4 | M | M | P | P | O | O | N | N |
| 1 | C | C | A | A | D | D | B | B |
| 5 | T | T | R | R | Q | Q | S | S |

**Note:** The same colour and letter indicate the number of observations per treatment level. Repeating numbers indicate the number of observations per treatment. The definition of letters is stated in Table 2 below.

**Table 2. In vitro concentrations of toxigenic and atoxigenic fungi and biochar of the experiment**

| S/N | Treatment | Level 1 | Level 2 | Level 3 | Level 4 |
|---|---|---|---|---|---|
| 1 | Atoxigenic A. flavus | A. $2\times10^2$ | B.$2\times10^4$ | C.$2\times10^6$ | D.$2\times10^8$ |
| 2 | Bch only | M. 20% Bch | N. 15% Bch | O. 10% Bch | P. 5% Bch |
| 3 | Atoxigenic A. flavus+Bch | E. (A+ P) | F.(B+O) | G.(C+N) | H.(D+M) |
| 4 | Atoxigenic A. flavus+Bch | I.(A+M) | J.(B+N) | K.(C+O) | L.(D+P) |
| 5 | Toxigenic A. flavus fungi | Q. (A+$2\times 10^2$) | R. (B+$2\times 10^{4)}$ | S.(C+$2 \times 10^6$) | T.(D+$2 \times 10^8$) |

Bch=Biochar, *A. flavus=Aspergillus flavus*.



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2.3.3 **Groundnut grain preparation and Inoculation**

Shelled groundnut grains were sterilized in a clean bowl using 70% ethanol and then rinsed with distilled autoclaved water three times to remove excess alcohol. After that, the sterilized grains were soaked in the solution of different concentrations of Biochar. Afterwards, 20 grains were placed onto wetted autoclaved tissue in the petri-dish base and arranged around the petri-dish base. Inoculation of 10 µl toxigenic fungi ($2 \times 10^6$) onto groundnut grains in the Petri dishes for the different concentrations of atoxigenic spore suspension and Biochar was done using a micro-pipette.

Thereafter, the petri dish was incubated for seven days at a temperature of $30^0$C and a relative humidity of 94±2%. The negative control was inoculated with toxigenic A. *flavus* without Biochar. In each treatment, there were two observations. After seven days, samples were dried at the temperature of $50^0$C for six hours, ground, packed and stored in the refrigerator at the temperature of $4^0$C, waiting for aflatoxin quantification using an HPLC machine.

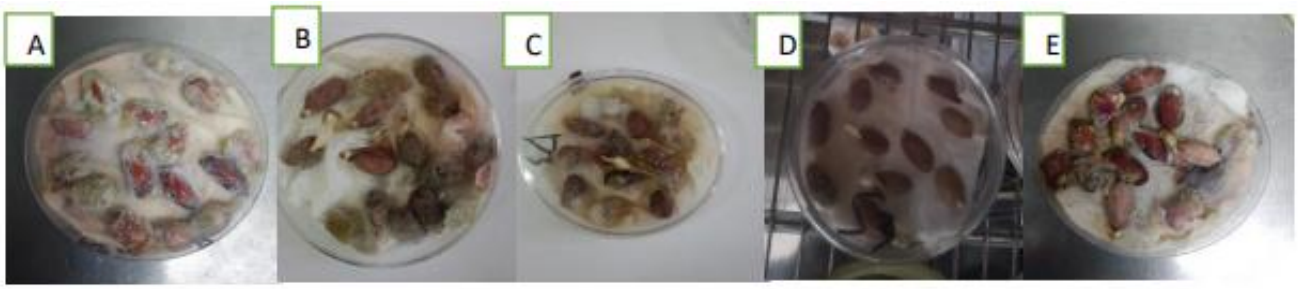


**Figure 3. Infected groundnut grains after seven days of incubation**

**Note:** (A) Negative control, (B) Inoculated grains rinsed in 5% Biochar, (C) Inoculated grains rinsed in 10% Biochar, (D) Inoculated grains rinsed in 15% Biochar, and (E) Inoculated grains rinsed in 20% Biochar.

**2.3.5 Aflatoxin Quantification Using High-performance Liquid Chromatography**

**(i) Sample preparation**

Groundnut grains were ground using a heavy-duty blender before 1.0g of the powder was taken and mixed with 5 ml of 70% methanol (v/v). Afterwards, the mixture was vortexed for 5 minutes and incubated at room temperature with shaking for 60 minutes. Thereafter, 10 minutes of Centrifugation at 3000×g was done to get the supernatant, which was used to quantify Aflatoxin.

**(ii) Extraction of Aflatoxin**

A 50ml polypropylene centrifuge tube was used to measure 5.0g of the sample, and then 1.0g of NaCl salt was added to it. The weighing spatula was sterilized with 70% ethanol and wiped dry with a paper towel after each sample. 25mL of 70% methanol was added into the 50-mL Falcon tube containing 5.0g of milled grains and NaCl, then shaken at room temperature for 20 minutes at 250 rpm. Twenty minutes later, samples were removed and allowed to stand undisturbed for 15 minutes. After that**,** samples extracted 1:1 were diluted with 1 % Acetic acid into 2 ml Eppendorf tubes, caped and vortexed for at least 10 seconds, and filtered through a GHP 0.2 µm syringe filter into a UPLC sample vial. The vial was caped and loaded into the UPLC autosampler for analysis. The concentration of Aflatoxins standard AFG1, AFG2, AFB1 and AFB2 were 50, 15, 50 and 15ng/mL, respectively. The column used was Phenomenex Synergi 2.5u Hydro – RP 100mm x 3.00mm. The mobile phase was Water: Methanol (60:40), and the flow rate was 0.4 ml/min. A standard calibration curve from a plot of peak areas against the known concentration of the injected volume was established using LabSolutions data analysis software. The injection volume was 20 µL for each. The retention time of the chromatographic peak of the target compound in the test sample and that of the corresponding standard chromatographic peak was used to identify the analyte of interest. The calibration curve was used to determine the concentration of the test solution. The values outside the linear range of the standard curve were re-analysed after being diluted and loaded into the UPLC autosampler. Note: Total Aflatoxin was the sum of the individual Aflatoxins.



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## 3. DATA COLLECTION

### 3.1 Aflatoxin content

Data on aflatoxin content in the test sample concentrations were calculated according to the formula below:

$$X\ (ng/g) = \frac{\mathrm{C} \times \mathrm{V} \times \mathrm{F} \times 100}{W \times \mathrm{R}}$$

*Where,*

$X$ – The overall content of distinct Aflatoxin in the test sample, ng/g

C – Aflatoxin concentration in the examined sample ( ng/mL),

V – Extraction volume (mL)

$F$ – Dilution factor

*100 – Recovery* Percentage

W – Test sample weight (g)

R - Recovery factor from spike recovery experiment

Note: All the aflatoxins data was in parts per billion (ppb)

## 4. DATA ANALYSIS

Data on Aflatoxin and soil nutrient contents were checked for normality (Shapiro–Wilk's test). Homogeneity of variances (Levene's test) and analyzed using analysis of variance (ANOVA), followed by mean separation test following Tukey's honest significant difference test ($P \leq 0.05$) using the JAMOVI statistical package version 2.3.2 (2022). Correlation analysis was done to measure the strength of the linear relationship between treatments and aflatoxin content and their association.

## 5. RESULTS AND DISCUSSION

The Analysis of Variance (ANOVA) conducted on various treatments revealed a significant difference ($P< 0.001$) among the means in reducing Aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ (Table 3). The mean separation using the Bonferroni test to assess the competitive ability of atoxigenic *A. flavus* and Biochar in reducing aflatoxin levels in groundnuts indicated significant ($P< 0.001$) differences for all treatments compared to the negative control. Integration of Aflasafe and Biochar at different rates exhibited slight differences, which were notably higher than the positive control (atoxigenic *A. flavus*) (Table 3). This outcome suggests that Biochar possesses the capacity to diminish the competitive ability of toxigenic *A. flavus*. A parallel observation was made by Iacomino *et al*. (2022), who demonstrated Biochar's capability to reduce the effectiveness of fungal pathogens by 86%. Additionally, several authors have reported on the positive effect of Biochar in reducing fungal pathogens' growth and infection in various crop grains, i.e. maize and tomato(Poveda *et al*., 2021). These findings can be attributed to Biochar's high surface area and numerous macro and micro-pores, as illustrated in Figure 2. This structure facilitates the absorption of cell-wall biodegrading enzymes released by *A. flavus* as an infection mechanism (Rahman *et al*., 2022), consequently enhancing the success of atoxigenic strains in competition.

The correlation analysis conducted in this study has unveiled intriguing insights into the relationship between treatments and aflatoxin content. When examining sole treatments with either Biochar or Aflasafe, a weak negative correlation with aflatoxin content was observed. However, the true power of Aflasafe and Biochar was revealed when combined. As depicted in Table 5, the synergy between Aflasafe and Biochar paints a compelling picture. Across the board, treatment combinations incorporating both treatments demonstrated superior efficacy compared to using Biochar or Aflasafe in isolation, a trend clearly evident in the data presented in Table 3. Among these combinations, the standout performer was the pairing of Aflasafe at a spore concentration of $2 \times 10^6$ conidia with a 10% Biochar application. This particular combination showcased an impressive 99.99% reduction in aflatoxin content, surpassing



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the efficacy of Aflasafe or Biochar alone, which achieved reductions of 90% and 87%, respectively (as indicated in Table 3). The rationale behind these findings likely lies in the influence of Biochar on the microenvironment inhabited by *A. flavus*. Indeed, previous studies, such as Mfaume *et al.* (2019), have highlighted the dependence of Biochar's effectiveness on factors such as pH, humidity, moisture, and temperature. For instance, it has been observed that high pH levels can suppress the expression of genes responsible for aflatoxin biosynthesis, thereby weakening the competitive ability of toxigenic *A. flavus*, as reported by Ivanova *et al.* (2016). The high pH of the Biochar (10.4), as shown in Figure 2, suggests its influence on the microenvironments surrounding groundnut grains. Toxigenic fungi thrive within a narrow pH range of 3.5-4.5, while atoxigenic strains can tolerate a wider pH range, which explains their success in competition in challenging conditions (Frenkel *et al.*, 2017). Similarly, Frenkel *et al.* (2017) have noted that Biochar fosters a soil microbiome conducive to the proliferation of natural enemies. Furthermore, Biochar could serve as a carrier for delivering both nutrients and microbial inoculants to agricultural soils (Kamali *et al.*, 2022). Its unique physical and chemical properties support the growth and activities of beneficial microbes, particularly by preventing desiccation during dry periods (Kamali *et al*., 2022). Traditionally, biochar has been utilized as a pesticide, as reported by Kochanek *et al*. (2022).

In essence, the combination of Aflasafe and Biochar presents a multifaceted approach to combating aflatoxin contamination, capitalizing on the complementary mechanisms of action of these two agents. This synergistic strategy not only enhances efficacy but also underscores the potential for integrated pest management solutions in addressing complex agricultural challenges.

In the present experiment, for example, when $2 \times 10^6$ conidia/petri dish of atoxigenic *A. flavus* were used in Biochar-free grains, the total aflatoxin level plummeted to 6.37ppb. However, employing the same concentration in grains treated with 10% Biochar resulted in an even more remarkable reduction, down to a mere 0.23ppb (Table 3). These findings hold significant implications for both food safety and crop protection, offering a promising solution to the enduring challenge of aflatoxin contamination in groundnuts.

The multiple linear regression analysis conducted in this study yielded valuable insights, indicating that each unit increase in Biochar, Aflasafe, and their integrated combination led to respective reductions in aflatoxin levels by 1.37, 2.03, and 5.6 ppb, maintaining a constant at 28.8 ppb (Table 6). These findings hold significant implications for the agricultural sector, suggesting that the strategic utilization of Biochar and Aflasafe can substantially mitigate aflatoxin contamination in groundnuts, thereby bolstering food and nutrition safety standards.

The study's findings shed light on a crucial relationship between aflatoxin contamination and the inoculum load of toxigenic *A. flavus*. What emerges is a compelling narrative revealing that as the concentration of atoxigenic *A. flavus* (Aflasafe) increases in the inoculated groundnut grains, the aflatoxin content decreases. This inverse correlation was starkly evident in the observations: when a 1:1 ratio of toxigenic to atoxigenic strains was employed, the aflatoxin content was measured at 6.37 ppb. However, with a shift to a 1:2 ratio, the aflatoxin content notably decreased to 4.27 ppb (as detailed in Table 3). These results resonate strongly with the findings of Jin *et al*. (2022), who similarly noted an escalating inhibitory effect of toxigenic *A. flavus* on aflatoxin production when multiple atoxigenic strains were introduced alongside a single toxigenic spore.

This discovery accentuates the pivotal significance of considering the initial concentration of toxigenic *A. flavus* inoculum when deploying Aflasafe biocontrol measures. Neglecting this factor could potentially compromise the efficacy of Aflasafe applications. However, the study provides a glimmer of hope by proposing a solution rooted in soil amendment with biochar before groundnut planting, a strategy supported by empirical evidence. Remarkably, when a 1:1 ratio was utilized in soil enriched with 10% Biochar, aflatoxin levels plummeted significantly to a mere 0.23 ppb. Even more encouragingly, when the ratio shifted to 1:2 under the same Biochar rate (as elucidated in Table 3).

Consequently, a notable gap in our understanding of the intricate dynamics governing the competition between toxigenic and atoxigenic *A. flavus* strains, particularly in relation to Biochar, comes to the forefront. This lacuna presents a fertile ground for further research and refinement of protocols aimed at enhancing the efficacy of aflatoxin control strategies. In essence, the study not only unveils a compelling narrative of cause and effect but also beckons



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towards a future where Biochar-amended soils could potentially serve as a robust bulwark against aflatoxin contamination in agricultural ecosystems. In essence, while significant strides have been made in unravelling the complex interactions between microbial populations and aflatoxin contamination, there is still considerable potential for enhancing our protocols and strategies for mitigating aflatoxin risk in agricultural contexts.

**Table 3.** Effect of different *A. flavus* inoculum and Biochar concentrations on Aflatoxin contamination in groundnuts.

| Treatments | AFB1 | AFB2 | AFG1 | AFG2 | TOT. AF |
|---|---|---|---|---|---|
| AFL.2×$10^6$+BCH. 10% | 0.1 a | 0 a | 0.11 a | 0.0233 a | 0.23 a |
| AFL. 2× $10^8$+ BCH5% | 0.15 a | 0.15 a | 0.0233 a | 0.0233 a | 0.34 a |
| AFL. 2× $10^8$+ BCH20% | 1.03 a | 0.02 a | 0.1133 a | 0.0233 a | 1.19 a |
| AFL. 2× $10^6$+ BCH15% | 1.49 a | 0 a | 0.05 a | 0 a | 1.54 a |
| AFL. 2× $10^4$+ BCH15% | 1.64 a | 0.093 a | 0.13 a | 0.0333 a | 1.9 a |
| AFL. 2× $10^4$+ BCH10% | 1.69 a | 0.68 a | 0.02 a | 0.0133 a | 2.41 a |
| AFL. 2× $10^2$+ BCH20% | 2.74 a | 0.787 a | 0.24 a | 0.0167 a | 3.79 ab |
| AFL. 2 × $10^2$+ BCH5% | 3.11 a | 0.427 a | 0.0433 a | 0.8133 ab | 4.39 abcd |
| AFL. 2 × $10^8$ | 4.15 ab | 0.047 a | 0.08 a | 0 a | 4.27 abc |
| AFL. 2 × $10^6$ | 6.1 abc | 0.207 a | 0.0667 a | 0 a | 6.37 abcde |
| BCH. 5% | 9.67 bc | 0.33 a | 0.2267 a | 0.3867 ab | 10.61 bcde |
| BCH. 15% | 10.07 bc | 1.367 a | 1.9367 c | 0.0563 a | 13.43 e |
| BCH. 10% | 10.19 bc | 0.87 a | 0.7767 ab | 0.17 a | 12.01 de |
| AFL. 2 × $10^4$ | 11.11 c | 0.257 a | 0.0233 a | 0.17 a | 11.56 cde |
| BCH. 20% | 25.96 d | 2.673 a | 0.3467 a | 0.91 ab | 29.89 f |
| AFL. 2 × $10^2$ | 28.97 d | 1.677 a | 0.3 a | 0.6 ab | 31.55 f |
| FNG. 2 × $10^2$ | 67.1 e | 13.21 b | 5.5533 e | 3.0133 c | 88.87 g |
| FNG. 2 × $10^4$ | 96.49 f | 27.713 c | 2.34 cd | 3.5533 cd | 130.1 h |
| FNG. 2 × $10^6$ | 235.38 g | 45.08 d | 3.13 d | 4.4633 d | 288.06 i |
| FNG. 2 × $10^8$ | 267.54 h | 51.55 e | 1.4267 bc | 1.6633 b | 322.18 j |
| CV% | 4.8 | 17.5 | 36.7 | 49.4 | 4.9 |
| LSD | 3.123 | 2.128 | 0.5135 | 0.649 | 3.892 |
| P-Value | <.001 | <.001 | <.001 | <.001 | <.001 |
| DF | 40 | 40 | 40 | 40 | 40 |

**Note:** AFL=Atoxigenic fungi concentration, BCH=% Biochar, FNG=Toxigenic fungi concentration, AF= Aflatoxin, LSD=Least significant difference, DF= Degrees of freedom. AFB1=Aflatoxin B1, AFB2=Aflatoxin B2, AFG1=Aflatoxin G1, AFG2=Aflatoxin G2, TOT.AF=Total Aflatoxin. The same letters indicate no statistical difference between and within the treatments. The unit of measurement was Ppb=parts per billion.



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**Table 4. Kendall's rank correlation coefficients between Biochar and Aflatoxin in groundnuts**

| | | | | | | |
|---|---|---|---|---|---|---|
| **AFB1** | **1.0000** | | | | | |
| **AFB2** | 0.0909 | 1.0000 | | | | |
| **AFG1** | 0.6667 | 0.1818 | 1.0000 | | | |
| **AFG2** | 0.5152 | 0.2121 | -0.4848 | 1.0000 | | |
| **Bch** | -0.134 | -0.9045 | -0.201 | -0.134 | 1.0000 | |
| **TOT. AF.** | 0.5344 | 0.5038 | 0.1985 | 0.4733 | -0.5232 | 1.0000 |
| | **AFB1** | **AFB2** | **AFG1** | **AFG2** | **BCH** | **TOT.AF.** |

AFB1=Aflatoxin B1, AFB2=Aflatoxin B2, AFG1=Aflatoxin G1, AFG2=Aflatoxin G2, TOT.AF=Total Aflatoxin, Bch=Biochar

**Table 5. Kendall's rank correlation coefficients between the combination (Biochar-Aflasafe) and Aflatoxin in groundnuts**

| | | | | | | |
|---|---|---|---|---|---|---|
| **AFB1** | **1.000** | | | | | |
| **AFB2** | 0.772 | 1.000 | | | | |
| **AFG1** | 0.732 | 0.394 | 1.000 | | | |
| **AFG2** | 0.047 | 0.178 | 0.106 | 1.000 | | |
| **Comb.** | -0.664 | -0.604 | -0.854 | -0.100 | 1.000 | |
| **TOT.AF** | 0.993 | 0.804 | 0.739 | 0.011 | -0.754 | 1.000 |
| | **AFB1** | **AFB2** | **AFG1** | **AFG2** | **Comb.** | **TOT.AF** |

AFB1=Aflatoxin B1, AFB2=Aflatoxin B2, AFG1=Aflatoxin G1, AFG2=Aflatoxin G2, TOT.AF=Total Aflatoxin, COMB=Combination of Atoxigenic A. Flavus and Biochar

**Table 6 Multiple Linear Regression showing the influence of Biochar, Aflasafe and their integration in Aflatoxin contamination in groundnuts in vitro**

| **Parameter** | **Estimate** | **s.e.** | **t pr.** |
|---|---|---|---|
| **Constant** | 28.88 | 2.83 | <.001 |
| **Biochar** | -1.372 | 0.207 | <.001 |
| **Aflasafe** | -2.031 | 0.192 | <.001 |
| **Comb(AFL+Bch)** | -5.704 | 0.103 | <.001 |

AFL=Aflasafe, Bch=Biochar, s.e= Standard erroe, t pr.= t probability

## 6. CONCUSSION

The findings of the present study unveil a promising avenue for tackling aflatoxin contamination through the synergistic action of Biochar and Aflasafe biocontrol measures. It emerges that Biochar plays a pivotal role in enhancing the efficacy of Aflasafe, offering a potent means of mitigating aflatoxin contamination in agricultural settings. One intriguing mechanism proposed is the potential of Biochar-coated seeds to diminish aflatoxin levels by absorbing cell wall degrading enzymes released by toxigenic *A. flavus*. Moreover, Biochar's ability to modulate the pH in the microhabitat surrounding toxigenic *A. flavus* roots further amplifies its impact, directly impeding aflatoxin biosynthesis gene expression and subsequent toxin production.

The study underscores a critical balance in Biochar's effectiveness, revealing its beneficial effects at lower concentrations while cautioning against its detrimental impact at higher doses, as evidenced in vitro experiments. This nuanced understanding suggests that Biochar, when integrated judiciously, can bolster Aflasafe efficacy and curtail the competitive prowess of toxigenic A. flavus, particularly at lower concentrations. Notably, the integration of



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Biochar and Aflasafe at specific rates (10% and 2 x $10^6$), respectively, emerges as a promising strategy for effective aflatoxin management.

The results further elucidate how Biochar amendment influences multiple facets of the aflatoxin contamination process in groundnuts. By affecting host susceptibility, fungal toxigenicity, and the microenvironment of the *A. flavus* toxigenic strains, Biochar emerges as a multifaceted tool in the arsenal against aflatoxin contamination. However, the study underscores the need for further investigation to unravel the long-term implications of Biochar employment as an aflatoxin management practice in open fields. Such inquiries hold the key to scaling up this promising approach and gaining a deeper understanding of the nuanced dose-effect relationship between Biochar and various crops.

In essence, the study not only elucidates the intricate interplay between Biochar and Aflasafe but also paves the way for innovative strategies in aflatoxin management, offering hope for safer and more sustainable agricultural practices.

## 7. Competing interests

The authors declare that they have no competing interests.

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## Availability of Data and Materials

Data will be available upon reasonable request to the corresponding author.

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