

Atoxigenic Isolates of *Aspergillus flavus* Effectively Reduce Cyclopiazonic Acid in a Sorghum-Based Matrix under Simulated Abiotic Stress Conditions

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Running head title: Atoxigenic *Aspergillus flavus* reduces CPA contamination

Impact statement: Mycotoxin contamination threatens food security and safety in many regions, particularly in the tropics and subtropics. While atoxigenic *Aspergillus flavus* isolates are widely used to reduce aflatoxins in staple crops, their potential to mitigate cyclopiazonic acid (CPA) remains unknown. This study demonstrates that atoxigenic isolates from Burkina Faso effectively suppress CPA production under abiotic stress conditions. Findings highlight the potential of biocontrol strategies to combat multiple mycotoxins simultaneously, reinforcing their importance in sustainable agriculture. By expanding biocontrol applications, this research supports safer food production and enhances resilience against mycotoxin contamination in vulnerable regions.

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Abstract

Maize, groundnut, and sorghum are important staple crops in several countries, but are prone to mycotoxin contamination. In the tropics and subtropics, *Aspergillus flavus* frequently contaminates those crops with aflatoxins and, sometimes, with cyclopiazonic acid (CPA). However, some genotypes cannot produce one or both toxins. In various countries, atoxigenic isolates of *A. flavus* are formulated into biocontrol products for field use to outcompete aflatoxin producers. The products effectively limit aflatoxin but their utility to reduce CPA remains unexplored. The abilities of four atoxigenic isolates (AF-) from Burkina Faso to control CPA by an isolate with high capacity to produce aflatoxins (AF+) and CPA was tested in co-inoculations at varying ratios (100+, 75+/25-, 50+/50-, 25+/75-, 100-), under simulated abiotic stress conditions. Experiments were conducted on 2% sorghum-based media at 0.95 and 0.90 water activity (a_w), at 30°C and 37°C, for 12 days. CPA was quantified using LC-MS/MS. CPA concentrations gradually decreased as the proportion of atoxigenic isolates increased, with effectiveness varying depending on the environmental conditions.

Keywords: mycotoxins, resilience, abiotic factors, biocontrol, bioprotectant, grains.

Introduction

The world population is growing at a high rate and there is the need to increase production and productivity of staple crops (Silva et al. 2023). However, in tropical and subtropical regions, some staples such as maize, groundnut, and sorghum are frequently contaminated with mycotoxins, including aflatoxins produced by *Aspergillus* section *Flavi* (Bandyopadhyay et al. 2007; Maina et al. 2016; Sserumaga et al. 2020). Aflatoxins are highly toxic and carcinogenic compounds that negatively affect health, trade, and income (Wu, 2015; Ismail et al. 2021). Thus, food safety strategies should be incorporated into efforts that aim to increase crop production and productivity.

A report made 2 decades ago stated that around 4.5 billion people in low- and middle-income countries (LMICs) are frequently exposed to aflatoxins (Williams et al. 2004). In sub-Saharan Africa (SSA) countries, aflatoxin contamination is a major food safety hazard, with chronic aflatoxin exposure and aflatoxicosis outbreaks reported over the years (Meijer et al. 2021). The capacity of countries to deal with mycotoxin challenges differs markedly based on their economic resources. High-income countries (HICs) enforce strict legislations for various food safety parameters for both locally produced and imported crops, resulting in relatively low mycotoxin exposure (Cheli et al. 2014; Postma et al. 2023). In contrast, in most LMICs several technological, institutional, and policy barriers prevent the effective enforcement of food safety legislations, and this results in high human exposure to various pathogens and contaminants (Bandyopadhyay et al. 2016; Matumba et al. 2017; Chilaka et al. 2022).

Food security globally is being adversely impacted by climate change (CC) (Wheeler and Von Braun 2013). Food safety is affected as well. More frequent and severe mycotoxin contamination events are expected (and are already occurring) because of CC (Medina et al. 2015; Battilani et al. 2016; Leggieri et al. 2021). Production of aflatoxins, especially aflatoxin B1 (AFB1), is usually triggered by environmental stresses, including water availability (Magan and Aldred 2007). Another mycotoxin produced by certain *Aspergillus* genotypes is cyclopiazonic acid (CPA) (Chang et al. 2009). Although not as potent as aflatoxins, CPA targets the nervous system and can cause gastrointestinal disorders (Ostry et al. 2018). While no regulated tolerance thresholds exist for CPA, this toxin contaminates a range of food and feed materials but has received less attention for developing strategies to control it.

Several technologies can limit aflatoxin contamination (Ojiambo et al. 2018). One of those is based on field use of atoxigenic (i.e., unable to produce aflatoxins) isolates of *A. flavus* as active ingredients in biocontrol formulations to competitively displace aflatoxin producers (Dorner 2004; Cotty 2006; Mehl et al. 2012). Use of atoxigenic-based biocontrol products results in crops with little to no aflatoxin (80 to 100% less) compared to adjacent, untreated crops. This technology was pioneered in the US by the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) and has been extended to several other geographies (Moral et al. 2020). Various biocontrol products under the trade name Aflasafe have been developed by the International Institute of Tropical Agriculture (IITA) and USDA-ARS along with national and international partners (Bandyopadhyay et al. 2016). To date, 16 Aflasafe products have been registered with regulatory authorities and licensed to partners for manufacturing and/or distribution.

Aflatoxin biocontrol has been repeatedly shown to effectively reduce aflatoxin in crops grown in thousands of farmers' fields under diverse cropping systems—including in hot, dry areas—in various SSA countries (Bandyopadhyay et al. 2022). The use of aflatoxin biocontrol, however, has raised concerns on whether the production of crops with low aflatoxin levels may promote increased concentration of other toxins. Research has shown that atoxigenic biocontrol treatment does not increase fumonisin levels in maize (Mauro et al. 2018; Ortega-Beltran et al. 2020; Reis et al. 2020). However, it is unknown if atoxigenic isolates used in biocontrol formulations promote higher CPA levels, regardless of their own toxin-producing ability. Although a recent criterion to select aflatoxin biocontrol isolates was the presence of defects in genes needed for both aflatoxin and CPA production (Moral et al. 2020), this criterion was not applied earlier during biocontrol development, and some isolates used in biocontrol formulations produce CPA. It has been argued that active ingredient fungi that produce CPA may contribute to high CPA exposure or that even if not producing it, could enhance CPA production by other fungi co-infecting treated crops (Ehrlich 2014; Ehrlich et al. 2015). However, there are no reports of increased CPA in crops grown in fields treated with biocontrol.

Laboratory assays have shown that i) atoxigenic isolates reduce aflatoxin content when co-inoculated both at equal and disadvantaged ratios with highly toxigenic fungi (Mauro et al. 2015), and ii) factors such as temperature and water activity (a_w) influence the success in limiting aflatoxin (Mohale et al. 2013; Medina et al. 2017). Although aflatoxin biocontrol products are not designed to control CPA, evaluating the abilities of atoxigenic fungi to limit CPA accumulation would be valuable. Therefore, the current study aimed to evaluate the effectiveness of four atoxigenic isolates of *A. flavus* from Burkina Faso, which produce neither aflatoxins nor CPA, in

controlling CPA produced by a highly toxigenic isolate of *A. flavus*. The experiments were conducted under interacting abiotic factors of temperature (30°C and 37°C), water stress (0.90 and 0.95 a_w), and at varying ratios in co-inoculations made in sorghum-based media. Such environmental conditions were selected to simulate abiotic stresses predicted to become more frequent under climate change scenarios, particularly in tropical and subtropical regions where sorghum is a key staple crop (Hossain et al., 2022). The selected temperatures reflect moderate (30 °C) and elevated (37 °C) thermal conditions that are increasingly common or projected under climate change scenarios. Similarly, the water activity levels represent mild (0.95 a_w) and more severe (0.90 a_w) drought stress conditions, simulating future environmental stresses that could impact fungal growth and mycotoxin production dynamics.

Materials and methods

Fungal isolates

Five isolates of *A. flavus* were used in the experiments. Four of them are atoxigenic isolates (AF-) recovered from groundnut (G018-2) and maize (M011-8, M109-2, M110-7) grown in Burkina Faso. These four atoxigenic isolates are the active ingredients of the aflatoxin biocontrol product Aflasafe BF01, developed for use in Burkina Faso (Adhikari et al. 2016; Bonkougou et al. 2024). In addition, the four isolates lack the capacity to produce CPA (Bonkougou et al. 2024). The highly toxigenic isolate La3228 (AF+), originating from maize grown in Nigeria, was selected because of its well characterized high production of aflatoxins (Atehnkeng et al. 2008) and frequent use in diverse experiments at IITA. La3228 also has a high capacity to produce CPA (*unpublished*). The isolates are part of the fungal collection of Aflasafe Unit in IITA-Ibadan and were sent to Cranfield

University as agar plugs of sporulating cultures in 4-ml glass vials containing 2 ml sterile distilled water. The isolates were sent to the UK under appropriate permits granted by quarantine authorities of each country.

Preparation of media

Sorghum grains were purchased from a local store in Cranfield (United Kingdom). The grains were ground in a Waring blender (Waring Commercial, Torrington, CT, USA). Sorghum-based media was prepared with 2% milled flour and 2% technical agar (Oxoid Ltd., Basingstoke, Hampshire, UK). The water activity (a_w) of the media was adjusted to 0.90 and 0.95 with appropriate amounts of glycerol, which was used as the modifying solute because it is stable over a wide temperature range (Nakagawa and Oyama, 2019). The media was autoclaved at 121°C and 15 psi for 20 min. After cooling, aliquots of 1.5 ml were poured into each of 20 wells of a 24-well Nunc sterile plate (Nunc Ltd., Astwood, Newport Pagnell, UK) inside a sterile laminar flow cabinet.

Inoculation of treatment plates with *A. flavus* strains

To prepare the working conidial suspensions, all isolates were grown on 2% sorghum-based medium, prepared as above, for 7 days at 30°C. Conidia were harvested using a sterilized loop and combined with 10 ml sterile water containing 0.01% TWEEN®80 in a 25 ml bottle. Homogenized conidial suspensions were quantified using a haemocytometer (Helber Cell Counting Chamber, Avantor Science Central, UK) and a compound microscope, and diluted to a concentration of 1×10^6 conidia/ml. Then, suspensions of 1×10^6 conidia/ml with different ratios of toxigenic and atoxigenic isolates were made as follows: 100% AF+, 75% AF+/25% AF-, 50% AF+/50% AF-, 25% AF+/75% AF-, and 100% AF-. Ten µl of the suspensions with various ratios were inoculated, four replicates per treatment, into the 24-well plates at a_w of 0.90 and 0.95. Plates of

the same a_w level were placed in closed polyethylene bags and incubated at either 30°C or 37°C for 12 days.

CPA extraction

CPA extraction was performed following Cervini et al. 2024. Briefly, a surface sterilized cork-borer ($\varnothing$ 5 mm) was used to individually remove ~350 mg of medium with fungal growth from each replicate and treatment. The removed plug was placed in a pre-weighed 2 ml Eppendorf tube and kept at -20°C until extraction. CPA was extracted using a solution of acetonitrile, formic acid, and water (79:0.1:20, v:v:v) at the ratio of 1:4 (sample:solution) added to the Eppendorf tubes. Tubes were shaken for 90 minutes at 300 rpm at 25°C on a rotary shaker (miniShaker VMR, Leighton Buzzard, UK). Then, tubes were centrifuged (Centrifuge 5417S Eppendorf, Stevenage, UK) at 22,600 g for 10 min at 25°C. A 500 μ l aliquot of the supernatant was then transferred into a 2 ml Agilent amber vial and stored at -20°C until analysis.

CPA analysis by LC-MS/MS

CPA was analysed by liquid chromatography tandem mass spectroscopy (LC-MS/MS) equipment (Agilent SCIEX, Q trap 6500+, US). The LC-MS/MS equipment was set for chromatographic separation at 25°C using a Gemini C18-column; 150 $\times$ 4.6 mm (Agilent Poroshell® 120 EC-18, Phenomenex®, Torrance, CA, USA) equipped with C18 guard cartridge of 4 $\times$ 3 mm. The samples, standards, and blank were injected into the system at a flow rate of 0.6 ml/min. Then, 10 μ l supernatant was injected into the system where mobile phases consisting of A (methanol:water:acetic acid v:v:v, 10:89:1 + 0.385 g ammonium acetate) and B (methanol:water:acetic acid v:v:v, 97:2:1 + 0.385 g ammonium acetate) were used. Ultra-purified

HPLC grade chemicals from Labcon (Fisher Scientific, UK) and reverse osmosis purified water (Ultrapure Water System, Purelab, Elga, UK) were used for all solvents and reagents. Standards for CPA (Biopure Romer Labs, Austria) were included in each batch of samples to generate a 10-point standard curve, with concentrations ranging from 0.5 ng/g to 1000 ng/g. A linear regression was applied, and the quality of the fit was evaluated by the R^2 value which was $R^2 = 0.999$. Data acquisition was conducted with Analyst version 1.6.3 and CPA quantified using MultiQuantTM v2.0.2 (SCIEX, USA). The limit of detection (LOD:0.42 ng/g) and the limit of quantification (LOQ:1.14 ng/g) were calculated according to Malachová et al. 2014.

Statistical analysis

Statistical analysis was performed using Statistica (version 14.0.0.15, TIBCO Software Inc, USA). To assess the main effects of individual variables (isolates and ratios) and their interactions (isolates $\times$ ratios), a two-way analysis of variance (ANOVA) was conducted. Post-hoc comparisons were performed using Tukey's Honestly Significant Difference (HSD) test to identify significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) in CPA accumulation during co-inoculations compared to CPA production by the toxigenic isolate alone. Graphs were generated using Origin 2018 (version 9.5, OriginLab Corporation, USA).

Results and discussion

The current study evaluated the relatively resilience of four atoxigenic isolates of *A. flavus* native to Burkina Faso to limit CPA production by a toxigenic *A. flavus* isolate, under diverse temperature and a_w conditions. The results showed that while the individual effect of the atoxigenic isolates was significant ($P < 0.05$) only at 37°C/0.90 a_w , the ratio of toxigenic to atoxigenic isolates had a

highly significant ($P < 0.001$) impact on CPA production across all temperature and a_w conditions (Table 1). The toxigenic isolate when inoculated alone produced high CPA levels, ranging on an average from 45,600 ng/g to 412,000 ng/g at 37°C/0.90 a_w and 30°C/0.95 a_w , respectively (Figure 1). At the 75+/25- co-inoculation ratio, CPA production was not significantly different from the toxigenic isolate alone at 30°C/0.90 a_w and 30°C/0.95 a_w (Figure 1a,b) while at 37°C/0.90 a_w co-inoculations with isolates G018-2, M109-2 and M110-7 significantly reduced CPA levels ($p < 0.05$ and $p < 0.01$, respectively) (Figure 1c). Similarly, at 37°C/0.95 a_w , G018-2 also caused a highly significant reduction in CPA production ($p < 0.001$) compared to the toxigenic isolate (Figure 1d). When the ratios were equal (50+/50-) or favored the atoxigenic isolate (25+/75-), CPA production was significantly reduced. An exception was observed for the co-inoculation 50+/50- with the M011-8 isolate at 30°C/0.95 a_w , where CPA production was not significantly different from the control (179,300 ng/g vs 412,100 ng/g) (Figure 1b).

Although none of the four atoxigenic isolates possesses the capacity to produce CPA (Bonkougou et al., 2024), relatively low levels of CPA (range = 80 to 8,470 ng/g) were detected in 100- treatments, that is, when the atoxigenic isolates were inoculated alone. Analysis of CPA levels in non-inoculated sorghum revealed levels similar as those obtained in the 100- treatments, indicating natural contamination in the substrate used. Overall, all four atoxigenic isolates were effective in limiting CPA production by the toxigenic isolate under most conditions, though differences were observed among the tested isolates.

These four atoxigenic isolates are the active ingredient fungi of the aflatoxin biocontrol product Aflasafe BF01, a registered product with demonstrated effectiveness in limiting aflatoxin contamination of maize, groundnut, and sorghum grown across vast areas with largely contrasting

agroecological zones within Burkina Faso, Mali, Niger, and Togo (Bonkougou et al. 2024). Further field studies are required to determine if the active ingredient isolates would also be effective in field conditions, and under CC conditions, in limiting CPA production if challenged with fungi capable of producing this toxin.

Sorghum is a crop that can withstand drought and heat challenges posed by CC (Hossain et al. 2022). It is expected that sorghum-growing areas will expand in future in arid and semi-arid regions where other staple crops are underperforming. About two decades ago, sorghum grown in West Africa was considered relatively safe from aflatoxin contamination compared to other cereals (Bandyopadhyay et al. 2007), although recently aflatoxin levels in sorghum have been increasing (Falade et al. 2022). Thus, sorghum, despite its resilience to CC challenges, needs aflatoxin mitigation strategies. Most aflatoxin biocontrol research has focused on maize (Gasperini et al. 2019; Kaburi et al. 2025) and groundnut (Crosta et al. 2025; Senghor et al. 2019) through laboratory and field studies, while data on sorghum remains limited. Indeed, the first report of aflatoxin biocontrol in sorghum, which was highly effective, was recent (Bonkougou et al. 2024). The laboratory-based research reported herein suggests that use of the aflatoxin biocontrol product containing the tested atoxigenic isolates, which also do not produce CPA, would be effective in limiting both aflatoxin and CPA contamination in sorghum. There are concerns that atoxigenic-based biocontrol usage may have unintended consequences resulting in reduced accumulation of aflatoxin but increased concentrations of other toxins such as fumonisins or CPA, or their derivatives (Ehrlich 2014; Ehrlich et al. 2015; Moore 2021). To date, increased fumonisin content or higher fumonisin-producing fungi densities in maize receiving atoxigenic-based biocontrol treatment in diverse maize field cropping systems have not been observed

(Mauro et al. 2018; Ortega-Beltran et al. 2020; Reis et al. 2020). In addition, there are no reports of increased CPA accumulation or their derivatives in commercial crops receiving biocontrol treatment.

Conclusions

Results from the current study, although laboratory based, demonstrated that the production of CPA by toxigenic fungi is also reduced when challenged by active ingredient atoxigenic isolates in biocontrol formulations. It remains to be investigated whether CPA is controlled by atoxigenic isolates with an intact CPA biosynthesis cluster. Moreover, farmers' field studies are needed to evaluate the effectiveness of the product in limiting CPA in sorghum, maize and other aflatoxin-prone crops.

Dedication

The authors dedicate this paper to the memory of Prof. Naresh Magan, who initiated and led this work at Cranfield University before his untimely passing.

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Author contributions

Vanshika Sharma: writing - original draft, investigation; Carla Cervini: writing - original draft, writing – review and editing, data curation, formal analysis, visualization, supervision (supporting); Carol Verheecke-Vaessen: writing - original draft, supervision, formal analysis;

Ranajit Bandyopadhyay: writing – original draft, writing – review and editing, funding acquisition, conceptualization; Angel Medina: writing – original draft, funding acquisition, conceptualization, supervision; Alejandro Ortega-Beltran: writing – original draft, writing – review and editing, funding acquisition, conceptualization, data curation, visualization; Naresh Magan: writing – original draft, funding acquisition, supervision, conceptualization, methodology, investigation.

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Conflict of interest

The authors receive no direct financial benefit from the manufacturing and marketing of any of the aflatoxin biocontrol products mentioned in this article. The Aflasafe name is a Trademark of the International Institute of Tropical Agriculture (IITA). IITA used to manufacture and commercialize Aflasafe for use in Nigeria, Senegal, Burkina Faso, The Gambia, and Ghana. Manufacturing and distribution responsibilities have been licensed to the private or public sector. IITA charges a small licensing fee to manufacturers for use of the Aflasafe name and cost associated with technology transfer and technical backstopping. This fee is reinvested for providing reasonable technical support to the manufacturer and distributor when needed, to

develop new products/practices for more farmers to use the technology, as well as for further research to improve the aflatoxin biocontrol technology. A portion of the fee is also set aside for fulfilling reasonable Access and Benefit Sharing obligations related to the Convention on Biodiversity. A. Ortega-Beltran is employed by IITA. R. Bandyopadhyay is an Emeritus Scientist with IITA.

Data availability statements

All data is incorporated into the article.

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Table 1. Analysis of variance (ANOVA) results for cyclopiazonic acid (CPA) production by the toxigenic *Aspergillus flavus* isolate La3228 (100+) alone and in co-inoculation with atoxigenic isolates (G018-2, M011-8, M109-2, and M110-7) under four environmental conditions (30°C/0.90 a_w, 30°C/0.95 a_w, 37°C/0.90 a_w, and 37°C/0.95 a_w). The effects of inoculation ratio, isolate type, and their interaction on CPA production were tested separately for each condition. Significant effects are indicated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001); ns: not significant. Significant differences are shown in bold.

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Condition (T/a _w)	Source of variation	SS	DF	MS	P value	Significance
30°C/0.90 a _w	Interaction	4.04E+08	12	3.36E+07	P=0.881	ns
	Ratios	2.37E+10	4	5.92E+09	P<0.001	***
	Isolates	7.80E+07	3	2.60E+07	P=0.746	ns
	Residual	2.53E+09	40	6.33E+07		
30°C/0.95 a _w	Interaction	4.45E+10	12	3.71E+09	P=0.967	ns
	Ratios	1.28E+12	4	3.20E+11	P<0.001	***
	Isolates	2.99E+10	3	9.97E+09	P=0.406	ns
	Residual	4.01E+11	40	1.00E+10		
37°C/0.90 a _w	Interaction	3.79E+08	12	3.16E+07	P=0.237	ns
	Ratios	1.70E+10	4	4.24E+09	P<0.001	***
	Isolates	2.29E+08	3	7.64E+07	P=0.032	*
	Residual	9.45E+08	40	2.36E+07		
37°C/0.95 a _w	Interaction	1.00E+09	12	8.33E+07	P=0.810	ns
	Ratios	5.47E+10	4	1.37E+10	P<0.001	***
	Isolates	7.02E+08	3	2.34E+08	P=0.172	ns
	Residual	5.35E+09	40	1.34E+08		

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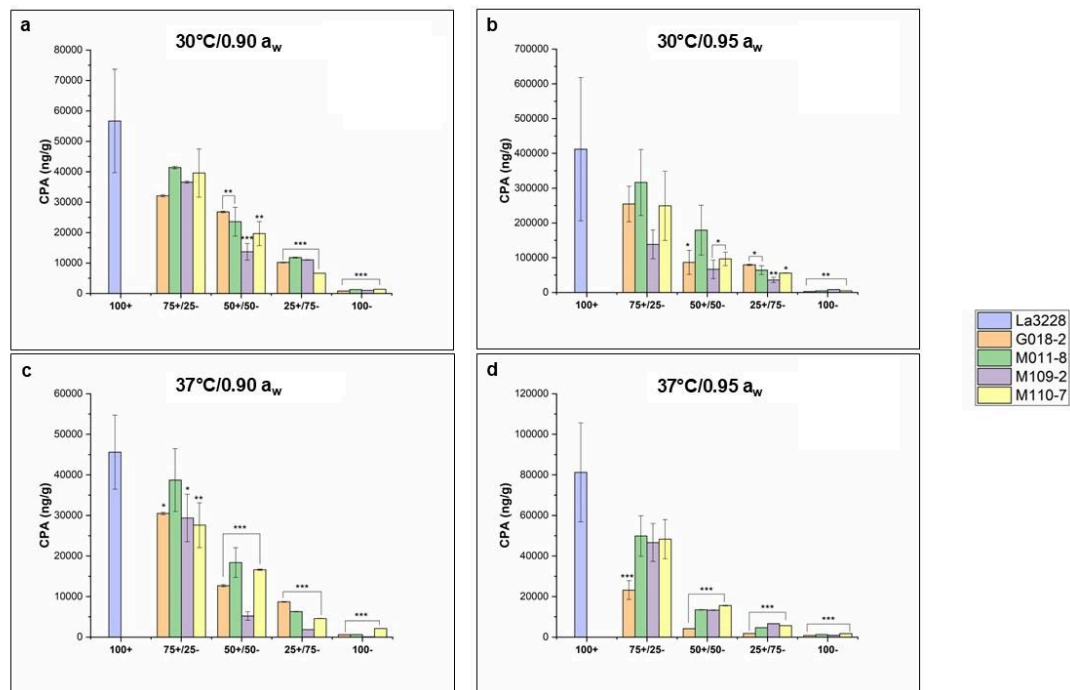
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Figure 1. Cyclopiazonic acid (CPA) production (ng/g) by the toxigenic *Aspergillus flavus* isolate La3228 (100+) alone and in co-inoculation with atoxigenic *A. flavus* isolates (G018-2, M011-8, M109-2, and M110-7) in sorghum-based medium, tested at varying ratios under four conditions: (a) 30 °C / 0.90 a_w, (b) 30 °C / 0.95 a_w, (c) 37 °C / 0.90 a_w, and (d) 37 °C / 0.95 a_w. The + and – signs indicate the relative proportions of the toxigenic and atoxigenic isolates in the spore suspension, respectively. Asterisks indicate statistically significant differences in CPA production compared to the toxigenic control (100+). Significance levels: * p < 0.05; ** p < 0.01; *** p < 0.001. Lack of asterisks indicate that no significant difference was observed.





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Atoxigenic isolates of *Aspergillus flavus* effectively reduce cyclopiazonic acid in a sorghum-based matrix under simulated abiotic stress conditions

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