

Impact of simulated climate change conditions on *Aspergillus flavus* biocontrol effectiveness in peanut-based medium and peanut seeds

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Abstract

Peanut is a legume widespread in the world, but its high susceptibility to *Aspergillus flavus* infection poses a significant challenge due to the risk of aflatoxin contamination. It is predicted that changing climatic conditions will result in warmer, drier periods with elevated CO₂ levels, which promote the growth of *A. flavus*. The most effective pre-harvest mitigation strategy is the use of non-aflatoxigenic strains of biocontrol; however, future climatic conditions may influence the effectiveness of this practice. Thus, the objective of this study was to assess the impact of simulated climate change conditions on the efficacy of a non-aflatoxigenic *A. flavus* strain, the active agent of a biocontrol product, in reducing fungal growth and mycotoxin production. A range of temperature conditions (T= 25, 30, 35°C), water activity (a_w= 0.85, 0.90, 0.95) and CO₂ concentration (400, 1000 ppm) were selected for investigation. The assay was conducted using three ratios of *A. flavus* spore suspensions (100% aflatoxigenic, 100% non-aflatoxigenic, 50/50% aflatoxigenic:non-aflatoxigenic; 10⁵ spores ml⁻¹) inoculated *in vitro* on a peanut-based medium (PBM), and *in situ* on peanut seeds, and incubated for 10 days. Results of *in vitro* studies showed a significant influence of T and a_w on fungal growth rates (μ), with a reduction when the a_w decreased and T shifted from the fungus's optimum of 30°C. The highest mycotoxin concentration was detected on PBM, with an aflatoxin B1 (AFB1) production by the aflatoxigenic strain *in situ* 50% lower than *in vitro*. However, for all the treatments, the application of the biocontrol agent inhibited AFB1 production with a general reduction of 55% *in vitro* and 71% *in situ*, even though a significant increase in kojic acid production was observed. The effectiveness of the non-aflatoxigenic strain increased when T was raised up to 35°C with higher AFB1 reductions both *in vitro* and *in situ*, of respectively 58 and 76%.

These observations provided the first evidence that climate change will not negatively influence the ability of the Italian *A. flavus* non-aflatoxigenic strain, which represents the biocontrol agent of the commercial product AF-X1, to reduce AFB1 contamination in peanuts.

Keywords: Mycotoxin; Aflatoxin B1; Temperature; Water activity; Carbon dioxide

1. Introduction

Peanut (*Arachis hypogaea*) is a legume spread in tropical and subtropical regions. Originally from South America, peanuts have spread all over the world for their great nutritional qualities that make them a very versatile product. The high content of lipids makes oil production one of the main uses of peanut seeds, but the richness in protein, vitamins, minerals, antioxidants, and other beneficial compounds, add to peanuts great health benefits (Arya et al., 2016). China and India are the world's largest producers and together represented over 50% of the 2022 total production of over 54 million tons (FAOSTAT, 2023). In Italy, the interest in this crop is growing and, in the last 10 years, the production has increased from 4.3 to 712.6 tons (Istat, 2024).

During the last decades, climate change (CC) raised a great deal of concern, not only for its direct consequences on the environmental conditions in various regions around the world but also for all the aspects that are influenced by these variations, i.e. food security and food safety. Focusing on Europe, the effect of climate change can be positive or negative, depending on the region. An average global temperature increases of 2°C or 4°C implies that certain regions will experience temperature rises exceeding 5°C or 6°C, but although the averages are lower in certain locations it might imply big changes (Battilani et al., 2016; Kos et al., 2023; Medina et al., 2017a).

In Northern and Central Europe, the increase in temperature and precipitation may lead to higher crop yields. However, in Southern Europe, crop production can be negatively affected by longer hot and drought periods caused by higher temperatures accompanied by a decrease in rainfall (Kos et al., 2023). These factors, associated with an atmospheric carbon dioxide (CO₂) concentration that is expected to double or triple in the future (from 417 ppm today to 800 ppm or 1000-1200 ppm), may have a significant effect on the growth of fungi on crops and foodstuff and the related mycotoxin contaminations (IPCC, 2023; Kos et al., 2023).

Several studies focused on the behaviour of various toxigenic fungi simulating climate change conditions of temperature (T), water activity (a_w), and CO₂, and showed that higher CO₂ level stimulated the growth and the mycotoxin production of fungi, in particular, when they underwent to stressful climatic conditions (high temperature and low a_w) (Baazeem et al., 2021a; Cervini et al., 2021; Medina et al., 2015; Verheecke-Vaessen et al., 2019).

One of the main effects of climate change on toxigenic fungi is the spreading of thermotolerant fungi, such as *Aspergillus flavus*, from the sub-tropical environments to the temperate ones, and many cases have been detected over the years (Battilani, 2016; Battilani et al., 2016). In 2003, due to the hot and dry summer, the maize produced in Northern Italy was subjected to the first significant aflatoxin contamination (Giorni et al., 2007; Piva et al., 2006). More recently, aflatoxin contamination occurred on Serbian maize caused by elevated temperatures and a drought period during the summer of 2012 (Kos et al., 2013). In addition to maize, there are other crops susceptible to *A. flavus* infection and consequent mycotoxin contamination, such as peanuts, pistachio nuts, and cottonseed (Horn, 2006; Magan et al., 2011).

Many studies were conducted to assess the optimal conditions for fungal growth and aflatoxin production and the impact of climate change on these characteristics (Baazeem et al., 2021a; Medina et al., 2017a). Baazeem et al. (2021a) assessed, on pistachio, the effect of the interaction of T (20°C, 25°C, 30°C, 35°C), and a_w (0.88, 0.90, 0.93, 0.95, 0.98, 0.995), on growth and aflatoxin B1 production by *A. flavus*. The results showed that the strains of *A. flavus* tested grow between 25 and 35°C. Whereas a higher impact of the interaction on abiotic factors has been recorded for aflatoxin production. The optimal conditions were 30 °C with 0.98 and 0.95 of a_w, for pistachio-based, whereas on pistachio nuts, the highest production was observed at 35°C with 0.98 a_w. These last conditions were interesting because the high temperature recorded highlights the ability of the fungus to tolerate higher temperatures than the normal optimal conditions (28-30 °C) (Giorni et al., 2007; Sanchis and Magan, 2004). A similar study was conducted by Medina et al. (2017a); in this research the authors, in addition to T and a_w, tested the effect of different CO₂ levels (350, 650, and 1000 ppm). The findings showed that the higher levels of CO₂ have a significant impact on aflatoxin production in drought stress conditions at 37°C. In particular, the contamination was significantly higher at 650 and 1000 ppm of CO₂ than at 350 ppm.

Various pre- and post-harvest strategies are used to prevent and manage aflatoxin contamination. Biocontrol, using non-aflatoxigenic strains of *A. flavus*, is one of the pre-harvest practices used to reduce aflatoxin

contamination of crops (Dorner, 2008; Mauro et al., 2018). Considering that this practice exploited the competition between non-aflatoxigenic and aflatoxigenic strains of *A. flavus*, it is important to understand the effect of the future forecast climatic conditions on the interactions between the non-aflatoxigenic and aflatoxigenic strains, and if these factors can influence the effectiveness of this practice. As shown by Magan and Lacey (1984), environmental factors, such as a_w , T and nutritive sources, influence the interactions between fungi, both intra- and inter-species (Magan and Lacey, 1984).

Over the last years, some studies have been conducted to examine the impact of aflatoxin contamination of non-aflatoxigenic strains of *A. flavus*, under different a_w , T, and CO₂ conditions (Gasperini et al., 2019; Medina et al., 2017b; Mohale et al., 2013). Up to now, in literature, the studies concerning climate change's impact on *A. flavus* biocontrol are focused on maize and AFB1 production.

However, *A. flavus* can produce a wide range of metabolites and the changing climatic conditions may influence their production. In general, aflatoxin B2, G1 and G2, kojic acid (KA), 3-nitropropionic acid (3-NPA), and sterigmatocystin (STC), are some of the most synthesised by *A. flavus* and may pose issues not only for their toxicity to humans and animals but also for adverse effects during processing, especially kojic acid (Kos et al., 2022; Navale et al., 2021; Wicklow, 1999).

The interest in Italian peanut production and the future environmental conditions in the Mediterranean region (Kos et al., 2023), highlights the need to delve deeper into the influence of climate change on the behaviour of *A. flavus* and the efficacy of biocontrol strategies on this crop. Therefore, this research aims to assess the impact of the interaction between three climate change-related factors as T, a_w , and CO₂ concentration on two *A. flavus* strains (aflatoxigenic and non-aflatoxigenic), in terms of i) fungal growth, ii) mycotoxin production, iii) biocontrol effectiveness of the non-aflatoxigenic strain, *in situ* on two varieties of peanuts kernels (Lotos and SIS_AR_01), and *in vitro* on a peanut-based medium.

2. Materials and methods

2.1. Aspergillus flavus strains and peanut varieties

One aflatoxin-producing strain of *A. flavus* (ITEM 8069) isolated from maize in north Italy (Giorni et al., 2007), and one non-aflatoxin-producing strain of *A. flavus* (MUCL54911), the active agent of AF-X1 (Mauro et al., 2018) have been selected for this study. Both strains were stored in the fungal collection of the Department of Sustainable Crop Production – section Crop Protection, Università Cattolica del Sacro Cuore, Piacenza; the latter is also deposited at Mycothèque de l'Université Catholique de Louvain (MUCL) under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure.

Two peanut varieties have been considered: Lotos is the most widespread variety in Italy, whereas SIS_AR_01 is a local Italian peanut variety. These varieties were produced in northern Italy in 2023 and they were supplied by the company Bonifiche Ferraresi S.p.A.

2.2. Inoculum production

The selected *A. flavus* strains were grown on Potato Dextrose Agar (PDA, Oxoid Ltd.) in 90-mm Petri dishes, at 30°C for 7 days in the dark. At the end of the incubation period, 10 mL of sterile distilled water was added to the plates to wash conidia that were then added to 25 mL of sterile distilled water, and the concentration was adjusted to 10⁵ conidia/mL. Subsequently, spores of aflatoxigenic and non-aflatoxigenic strains were mixed at a ratio of 50:50, to obtain an inoculum with equal quantity of both strains.

2.3. Environmental conditions and climate change system

To test the combined effect of three temperatures (T: 25, 30, 35 °C), three water activities (a_w : 0.85, 0.90, 0.95) and two carbon dioxide (CO₂) concentrations (400, 1000 ppm) 12-L air-tight environmental chambers, adapted with two valves (one inlet and one outlet), were used in this study. These were flushed daily for 10 days (3L/min) with air 400 ppm (current) and using synthetic gas mixture cylinders to simulate 1000 ppm

(increased) CO₂ (British Oxygen Company, Guildford, Surrey, UK). In particular, to obtain the 400 ppm the containers were left open for the same duration of the CO₂ flushing, to replace 3x the air volume of the environmental chamber for both the CO₂ conditions (Cervini et al., 2024). The temperature was controlled using incubators (SANYO Electric Co., Ltd., MLR-350H) at 25, 30, and 35°C, and the a_w was controlled within each chamber by adding 400 mL beakers with the right solution of glycerol-water to maintain the equilibrium relative humidity to the desired a_w levels (46 g, 101.2 g and 156.4 g of glycerol were added to 200 mL of water to have respectively 0.95, 0.90 and 0.85 of a_w).

2.4. Chemicals

LC grade acetonitrile and methanol, MS grade ammonium acetate, and acetic acid were provided by (ThermoFisher, UK). The Pure Lab Ultra system (ELGA LabWater, Celle, Germany) was exploited to obtain the pure water. Mycotoxin standards used in this work, such as aflatoxin mix B1, B2, G1, G2, kojic acid (KA), 3-nitropropionic acid (3-NPA), and sterigmatocystin (STC), were purchased from Biopure Romer labs, Austria.

2.5. *In situ* study

2.5.1. Sample preparation

Peanut kernels were surface sterilized by submersion in hot sterile distilled water for 45 seconds at 80°C (Mehl and Cotty, 2010), and then dried at room temperature in a biosafety cabinet (HeraSafe, Heraeus, Germany). After that, the kernels were put inside sterile glass flasks aseptically closed, to keep the sterility until the next step. Water activity was checked using the Aqualab 4TE water activity meter (Aqualab 4TE; Decagon Devices, Inc., Pullman, WA, USA). To assess the effectiveness of the sterilization treatment, 5 kernels were directly plated onto Potato Dextrose Agar (PDA) plates.

To modify the a_w level to targeted values of 0.85, 0.90, and 0.95 a_w a moisture adsorption curve for the shelled peanuts varieties used in this study, was developed. Twelve sub-samples of 5 g of peanut kernels were put into 25 ml glass bottles and different known amounts of water were added (0, 0.25, 0.50, 1.00, 1.25, 1.50 mL). After stirring and sealing, bottles were left overnight at 4°C, and then the a_w was measured using the Aqualab 4TE a_w meter. The results of these analyses were plotted and the relation between a_w and specific amounts of water was used to obtain the volume of water that needed to be added to the seeds to reach the target experimental conditions of a_w .

After sterilization, the peanut kernels were put in sterile glass flasks and the appropriate amounts of sterile water were added to adjust the a_w according to the moisture seed content (MSC), excluding the water of inoculum, and then they were left at 4°C overnight to equilibrate. Water activity was confirmed using the Aqualab 4TE water activity meter before inoculation.

2.5.2. Inoculation of peanut seeds

The inoculation of seed was carried out following the method of (Gasperini et al., 2019). Briefly, 10 grams of treated peanut seeds were put aseptically into glass culture vessels (Magenta™, Sigma, USA) and sealed with lids containing a gas-permeable membrane (10 mm with a polypropylene membrane 0.22 µm pore size). The seeds were inoculated with 100 µL of spore suspensions (10⁵ conidia/mL) with 50:50 aflatoxigenic and non-aflatoxigenic strains and shaken to evenly distribute the inoculum over the seeds. Three controls were included consisting of peanut seeds inoculated with each strain singularly (100% aflatoxigenic and 100% non-aflatoxigenic), and kernels inoculated with pure sterile water without conidia. Control samples were only considered at the optimal environmental conditions for fungal growth to evaluate the disinfection of kernels. In particular, two T (25, 30 °C), one level of a_w (0.95), and two CO₂ concentrations (400, 1000 ppm) were considered. The experiment was carried out in triplicate.

2.5.3. Mycotoxin extraction

After 10 days of incubation, peanut seeds were stored at -20°C until analysis. Afterwards, peanuts samples were dried for 24h at 60°C using an oven (Gen Lab Ltd., Model: OU/100/F). and then separately grounded using a grinder (Spice and nut grinder, SG20U, Cusinart), and stored at -20°C.

The extraction was carried out following Oyesigye et al. (2024). Briefly, one gram of each grounded sample was put in 15 ml falcon tubes where 4 ml of extraction solution (acetonitrile:water:acetic acid, 79:20:1, v/v/v) was added. Then samples were shaken at 3000 rpm for 90 minutes using a rotatory shaker (Heidolph, Multi Reax, 220-230 V, 50-60 Hz, Germany). The samples were centrifuged (Thermo Scientific, Heraeus Labofuge 400 R, Germany) for 2 minutes at 3000rpm; 350 µl of the resulting supernatants were added to amber glass vials together with 350 µl of elution solution (acetonitrile:water:acetic acid, 20:79:1, v/v/v), and the vials were stored again at -20°C until LC-MS/MS analysis.

2.5.4. LC-MS/MS analysis

The LC-MS/MS analysis was performed by the injection of 1 µl from each vial into the qTRAP 6500+ LC-MS/MS system (SCIEX instrument, Foster City, CA, USA) that was equipped with an electrospray ionisation (ESI). The column used in this experiment (*ACE 3-C18 column*, 2.1 × 100 mm, 3 µm particle size; Hichrom) had a guard cartridge (4 × 3mm, Gemini, Agilent), and it was maintained at 60°C. An amount of 0.385 g/L of ammonium acetate was added as a supplement to the mobile phase A (methanol:water:acetic acid v:v:v, 10:89:1 + 385g of ammonium acetate) and the mobile phase B (methanol:water:acetic acid v:v:v, 97:2:1 + 385g of ammonium acetate). The flow rate was 0.6 ml/min and the gradient lasted 21.5 minutes as follows: 0 min, 0% B and 0–2.0 min, 0% B; 2.0–5.0 min, 50% B; 5.0–14.0 min, 100% B and 14.0–18.0, 100% B; 18.0–19.0 min, 0% B and 19.0–21.50 min, 0% B. Each set of two daughter ions for every metabolite underwent monitoring through Multiple Reaction Monitoring (MRM) in both positive and negative modes following the methodology outlined by Malachová et al. (2014). The source conditions comprised a Curtain gas set at 40%, Medium Collision Gas, IonSpray voltage ranging from 4500 V to 5500 V, Temperature held at 400 °C, both ion sources maintained at 60 psi, and a consistent 10 V Entrance Potential applied to all compounds. The data acquisition process was conducted using Analyst® Data Acquisition version 1.6.3, while quantification was executed using MultiQuant™ version 3.0.3. Quantitative analysis was facilitated by standard curve solutions containing aflatoxins B1, B2, G1, G2, kojic acid (KA), 3-Nitropropionic acid (3-NPA), and sterigmatocystin (STC). The determination of the limit of detection (LOD) and limit of quantification (LOQ) was carried out according to Malachová et al. (2014). Regular participation in proficiency testing schemes has continuously verified the accuracy of the method (Malachova et al., 2015; Malachová et al., 2014).

2.6. In vitro study

2.6.1. Media preparation and inoculation

A peanut-based medium (PBM) made of ground peanut seeds and water at a 1:1 ratio was used for the *in vitro* study. The a_w of the medium was adjusted to 0.95, 0.90, and 0.85 a_w by adding the right amounts of glycerol to water. The solution glycerol-water, used to equilibrate the a_w , was shaken, added to the peanut powder, and then the a_w was measured using the Aqualab 4TE water activity meter (Aqualab 4TE; Decagon Devices, Inc., Pullman, WA, USA). PBM was sterilised through tyndallisation which is reported to have a minimum effect on the chemical and nutritional composition of peanuts (Lazzaro et al., 2013). Briefly, the medium was heated for 30 mins at 80°C and then cooled down, this process was repeated three times, and between heat treatments, the medium was incubated at 30°C to allow the germination of spores which would be eliminated with the following heating. After the tyndallisation, the a_w was confirmed using the Aqualab 4TE water activity meter. Twenty grams of PBM were poured into 90 mm Petri dishes closed in polyethylene bags, according to the a_w , and stored at 4°C until use.

A. flavus strains, aflatoxin producer and non-producer, were inoculated on PBM both individually at the centre and together at a distance of about 40 mm. The inoculum consisted of 10 µL of spore suspension (10^5

conidia/mL) for each strain. The control was represented by non-inoculated PBM. The experiment was performed in triplicate.

2.6.2. Fungal growth assessment and index of dominance (I_D)

Two perpendicular diameters of the colonies were daily measured during the whole incubation (10 days) using a ruler, and the average diameters were calculated. The diameters were then plotted against time and a linear regression model was used to obtain the lag phase (λ , days), and the growth rate (μ , mm/day) (Medina et al., 2015).

The type of interaction between the two strains was defined visually and a score was given to each strain according to (Mohale et al., 2013). In detail the interactions were classified as: mutual intermingling (score=1); mutual antagonism, when the colonies meet but they are mutually inhibited (score=2); mutual antagonism at a distance when the colonies are separated, they do not meet (score=3); dominance of one specie on contact (score= 4 for the dominant one and 0 for the inhibited one); dominance of one specie at distance (score= 5 for the dominant one and 0 for the inhibited one) (Giorni et al., 2014). The defined numerical scores, were then added to obtain an overall I_D which represent a measure of the competitiveness of *A. flavus* strains (aflatoxigenic vs. non-aflatoxigenic), under all the tested environmental conditions (Magan and Lacey, 1984).

2.6.3. Mycotoxin extraction

After 10 days, 6 agar plugs ($\varnothing = 4$ mm) for a total of about 350 mg were taken from each plate. When the aflatoxigenic and non-aflatoxigenic strains were co-inoculated, three plugs were collected for each colony (one from the centre and two from the external competing part of colonies), put in 2 ml pre-weighed Eppendorf tubes, and then stored at -20°C until the analysis. According to Cervini et al. (2024), an amount of four times the weights of the plugs of acetonitrile:water:acetic acid (79:20:1 v/v/v) was added to each tube and shaken for 90 mins at 300 rpm at 25°C on a rotatory shaker (miniShaker VMR, Leighton Buzzard, UK). Then, the tubes were centrifuged at 14000 rpm for 10 min (Centrifuge 5417S Eppendorf, Stevenage, UK), 350 μl of supernatant from each tube were transferred to amber glass vials with 350 μl of acetonitrile:water:acetic acid (20:79:1 v/v/v), and stored at -20°C until LC-MS/MS analysis (paragraph 2.5.4).

2.7. Statistical analysis

The statistical analysis was carried out using the statistical package IBM SPSS Statistics 27 (IBM Corp., Armonk, NY, USA). The analysis of variance (ANOVA) and Tukey's HSD *post-hoc* test ($\alpha = 0.05$) were performed to assess the normal distribution and significant differences, respectively of both fungal growth and mycotoxin data sets. Data about the mycotoxin production were $\ln$ transformed ($y = \ln(x+1)$) before analyses to homogenize the variance.

3. Results

3.1. In situ study

3.1.1. Mycotoxins production

Data about all the mycotoxins produced, by *A. flavus* after 10 days on peanut seeds, were analysed by applying the ANOVA test (table 1). Aflatoxin G1 and AFG2 were not considered, because of the low concentration, as in in vitro trials.

The influence of peanut variety was significant for the production of all the tested mycotoxins. In particular, Lotus showed significantly lower concentrations of AFB1, KA, and STC than the variety SIS_AR_01, which was characterized by a generally higher mycotoxin contamination.

The effect of the non-aflatoxigenic strain as a biocontrol agent led to a significant reduction of AFB1, AFB2, 3-NPA, and STC concentration (71, 78, 63, 76% respectively) in peanut seeds co-inoculated with the two *A.*

flavus strains. Unlike the others, KA concentration significantly increased when the aflatoxigenic strain was co-inoculated with the non-aflatoxigenic one.

Generally, a significantly higher mycotoxin production was observed at T= 30 °C, a_w = 0.95, and CO₂= 400 ppm, except for the 3-NPA which showed a significant increase at 25°C. Aflatoxin B1 production significantly decreased from 30°C to 25 and 35 °C, by 9% and 50 %, respectively. The influence of a_w was comparable among all the mycotoxins, with a significant reduction in the production when the drought level increased. Focusing on AFB1, the concentration on peanut seeds after 10 days was 43% and 85% lower when the a_w decreased, respectively from 0.95 to 0.90 and 0.95 to 0.85.

The interaction between inoculum and a_w , as well as inoculum x T, did not show any significant effect on mycotoxin production, except for the STC.

Table 1. Production of aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), kojic acid (KA), 3-Nitropropionic acid (3-NPA), and sterigmatocystin (STC) by aflatoxigenic strain alone and co-inoculated with the biocontrol strain, on peanut seeds of two different variety, under the effect of single and interactive CC-related factors. Results of the analysis of variance (ANOVA). Tukey's HSD post-hoc test ($\alpha=0.05$) has been carried out by factor.

<i>Factors</i>	<i>AFB1 ($\mu\text{g/kg}$)</i>	<i>AFB2 ($\mu\text{g/kg}$)</i>	<i>KA ($\mu\text{g/kg}$)</i>	<i>3-NPA ($\mu\text{g/kg}$)</i>	<i>STC ($\mu\text{g/kg}$)</i>
Variety	**	**	**	**	**
Lotus	1539.4 b	552.2 a	27.8 b	0.74 a	4.8 b
SIS_AR_01	1666.2 a	446.3 b	38.4 a	0.21 b	7.4 a
Inoculum	**	**	**	**	**
Aflatoxigenic alone	2490.6 a	820.1 a	20.6 b	0.70 a	9.9 a
Co-inoculum	715.0 b	178.5 b	45.6 a	0.26 b	2.3 b
Temperature (°C)	**	**	**	**	**
25	1815.0 b	661.5 a	23.6 b	1.22 a	6.8 a
30	1995.9 a	574.6 a	49.1 a	0.16 b	9.2 a
35	997.5 b	261.7 b	26.6 b	0.05 b	2.3 b
Water activity	**	**	**	**	**
0.85	436.1 c	127.8 c	11.7 c	0.30 b	2.2 c
0.90	1526.7 b	516.0 b	35.8 b	0.04 b	6.0 b
0.95	2845.6 a	854.0 a	51.8 a	1.09 a	10.2 a
CO₂ concentration (ppm)	**	**	**	NS	**
400	1827.2 a	541.9 a	45.0 a	0.65	7.9 a
1000	1378.3 b	456.6 b	21.1 b	0.30	4.3 b
Interaction					
Inoculum x Temperature	NS	NS	NS	NS	NS
Inoculum x Water activity	NS	NS	NS	NS	*

***" $P<0.01$; "**" $P<0.05$; "NS" not significant; different letters indicate significant differences according to Tukey's HSD test.

3.2. In vitro study

3.2.1. Effect of climate change factors on I_D and fungal growth

The effect of T, a_w , and CO₂ on the interaction scores between the fungal strains are shown in Table 2. Some conditions led to minimal or no growth, and the interaction was not classified.

In general, the environmental conditions affected the interaction with a decrease in competitiveness when T and a_w increase under $\text{CO}_2 = 400\text{ppm}$. Indeed, the strains moved from mutual antagonist at a distance at $T=25^\circ\text{C}$ and $a_w=0.90$, to mutual intermingling at $T=30$ and 35°C , and $a_w=0.90$. In contrast, when the CO_2 concentration increased (1000 ppm), the strains became more competitive at the highest T (35°C). In particular, the non-aflatoxigenic strain was more dominant at $T=35^\circ\text{C}$ and $a_w=0.85$.

CC-related factors affected the growth of *A. flavus*. The individually inoculated aflatoxigenic strain showed a μ significantly lower than the non-aflatoxigenic one, with a reduction of 12%; when the strains were co-inoculated, their growth significantly decreased compared to their respective single inoculation, but they were comparable. However, there was a significant reduction of μ in the co-inoculum. Regarding λ , the best performances were observed when the strains grew together and the only significant difference was obtained between the aflatoxigenic strains alone and the co-inoculated ones.

Considering the T, at 30°C the fungus grew significantly faster than at 25°C and 35°C , with a μ reduction of 45% and 14% respectively, recording also a significant difference between the μ at 25°C and 35°C . The λ was significantly lower at the highest T (35°C) and it increased with the reduction of the T reaching the highest value at 25°C .

The driest simulated condition ($a_w=0.85$) led to worse growth performances, with a 54% significant reduction of the μ than at $a_w=0.90$, together with a λ increase of about 35%. The exposure of the colonies to a high CO_2 concentration (1000 ppm) did not have any impact on the μ ; nevertheless, the λ were significantly reduced.

The interaction between the CC-related factors (T x a_w) and the inoculum conditions (alone or co-inoculated) significantly affects μ and λ of the strains (table 3).

Table 2. Effect of temperature, water activity, and CO_2 on interaction scores and index of dominance (I_D), of *Aspergillus flavus* aflatoxigenic (AFL^+) and non-aflatoxigenic (AFL^-) strain on PBM.

		Temperature ($^\circ\text{C}$)								
CO ₂	Interacting strains	25			30			35		
		0.85	0.90	I_D	0.85	0.90	I_D	0.85	0.90	I_D
400	$\text{AFL}^+/\text{AFL}^-$	*	3/3	3/3	3/3	1/1	4/4	3/3	1/1	4/4
	I_D score	*	3/3	3/3	3/3	1/1	4/4	3/3	1/1	4/4
1000	$\text{AFL}^+/\text{AFL}^-$	*	3/3	3/3	*	1/1	1/1	0/5	2/2	2/7
	I_D score	*	3/3	3/3	*	1/1	1/1	0/5	2/2	2/7

“*” The interaction score was not calculated for minimal or no growth.

Table 3. *Aspergillus flavus* strains', single and co-inoculated on PBM, growth rate (μ) and lag phase (λ) under the effect of single and interactive CC-related factors. Results of the analysis of variance (ANOVA). Tukey's HSD post-hoc test ($\alpha=0.05$) has been carried out by factor.

Factors	μ (mm/day)	λ (days)
Inoculum	**	**
Aflatoxigenic alone	4.06 b	2.90 a
Aflatoxigenic co-inoculated	3.08 c	2.19 b
Non-aflatoxigenic alone	4.60 a	2.49 ab
Non-aflatoxigenic co-inoculated	3.34 c	1.90 b
Temperature (°C)	**	**
25	3.04 c	3.32 a
30	4.40 a	2.77 b
35	3.87 b	1.03 c
Water activity (a_w)	**	**
0.85	2.46 b	2.87 a
0.90	5.07 a	1.86 b
CO₂ concentration (ppm)	NS	**
400	3.68	2.67 a
1000	3.86	2.06 b
Interaction		
Inoculum x Temperature	**	**
Inoculum x Water activity	**	*

***" $P<0.01$; "**" $P<0.05$; "NS" not significant; different letters indicate significant differences according to Tukey's HSD test.

3.2.2. Mycotoxin production

The ANOVA analysis was applied to the data of all the mycotoxins produced on the PBM after 10 days of incubation (table 4). Regarding AFs, only the AFB1 and AFB2 were considered, AFG1 and AFG2 were negligible compared to the others.

The biocontrol led to a significant reduction of AFB1, AFB2, and STC, respectively 55%, 64%, and 94 % when the non-aflatoxigenic strain was co-inoculated with the aflatoxigenic one. However, a significant increase in KA in the co-inoculum was noticed.

Considering the main factors, the optimal conditions for mycotoxin production were $T=30^\circ\text{C}$, $a_w=0.90$, and $\text{CO}_2=1000$ ppm. The exception was represented by the KA, with the highest production at 25°C and 400 ppm. The statistical analysis confirmed a significant impact on mycotoxin concentration when the environmental conditions moved from the optimum. In particular, the a_w reduction caused a significant decrease of at least 70% in the dryest condition for all the mycotoxins.

3-Nitropropionic acid was observed at very low concentration and both inoculum and CC-related factors did not have any significant impact on its production.

The interactions between inoculum x T and inoculum x a_w were significant. The effect on AFB1 production, when the non-aflatoxigenic strain was applied, was significantly relevant at 30 and 35°C with reductions of respectively 55 and 59% (figure 1A). At the same time, the biocontrol agent led to a significant increase in KA at 30°C . In particular, the aflatoxigenic strain alone showed a KA production 80% lower than the co-inoculum condition (figure 1B).

Table 4. Production of aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), kojic acid (KA), 3-Nitropropionic acid (3-NPA), and sterigmatocystin (STC) by aflatoxigenic strain alone and co-inoculated with the biocontrol strain, on PBM, under the effect of single and interactive CC-related factors. Results of analysis of variance (ANOVA). Tukey's HSD post-hoc test ($\alpha=0.05$) has been carried out by factor.

<i>Factors</i>	<i>AFB1</i> ($\mu\text{g/kg}$)	<i>AFB2</i> ($\mu\text{g/kg}$)	<i>KA</i> ($\mu\text{g/kg}$)	<i>3-NPA</i> ($\mu\text{g/kg}$)	<i>STC</i> ($\mu\text{g/kg}$)
Inoculum	**	**	*	NS	**
Aflatoxigenic alone	4789.8 a	1122.7 a	146.5 b	0.09	9.2 a
Co-inoculum	2138.3 b	424.9 b	162.5 a	0.01	3.7 b
Temperature (°C)	**	**	**	NS	**
25	679.2 b	112.4 c	382.0 a	0.01	4.5 b
30	6402.5 a	1856.1 a	67.4 b	0.13	12.2 a
35	3361.3 a	349.8 b	7.5 c	0.01	2.7 c
Water activity (a_w)	**	**	**	NS	**
0.85	1197.4 b	180.8 b	15.9 b	0.01	2.8 b
0.90	5704.5 a	1360.0 a	288.9 a	0.09	10.1 a
CO₂ concentration (ppm)	*	*	*	NS	**
400	3283.4 b	681.5 b	204.8 a	0.01	3.2 b
1000	3676.5 a	873.2 a	105.3 b	0.09	9.7 a
Interaction					
Inoculum x Temperature	*	**	**	NS	**
Inoculum x Water activity	**	**	**	NS	**

“***” $P<0.01$; “**” $P<0.05$; “NS” not significant; different letters indicate significant differences according to Tukey's HSD test.

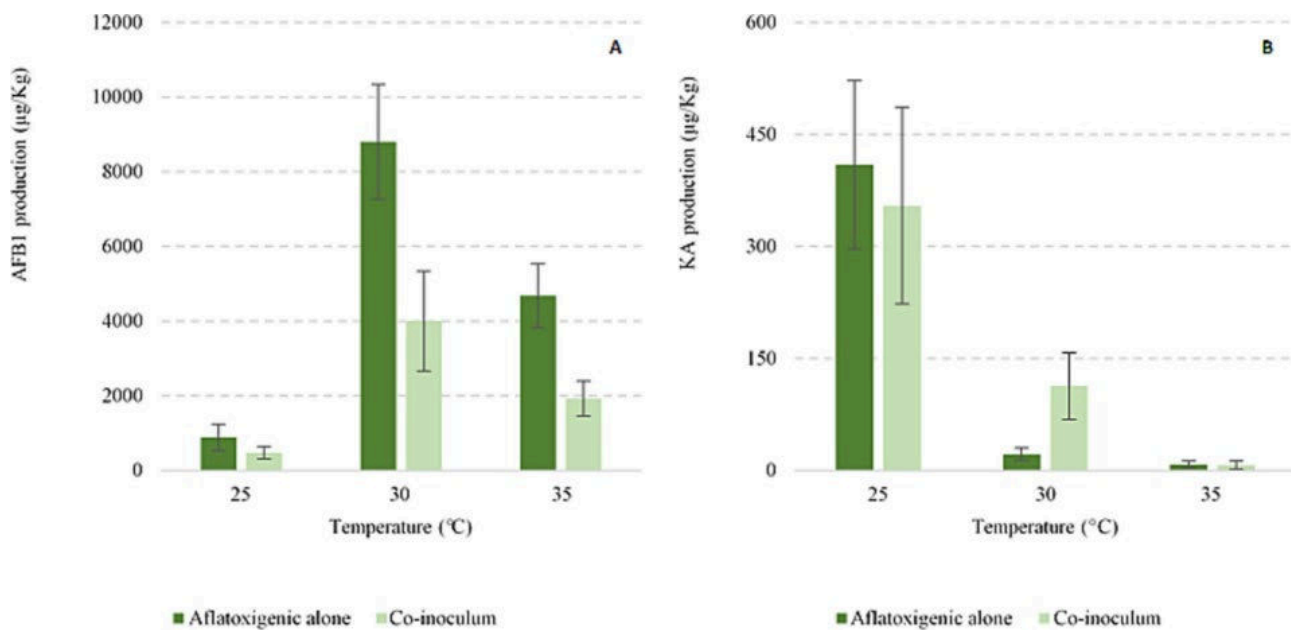


Figure caption

Figure 1. Production ($\mu\text{g/kg}$) of aflatoxin B1 (AFB1) (A) and kojic acid (KA) (B) by an aflatoxigenic strain of *A. flavus* grown alone or co-inoculated with a non-aflatoxigenic strain of *A. flavus*, at different T (25, 30, 35 °C), on peanut based medium. The bars indicate the mean standard error.

4. Discussion

Climate change impacts all living organisms, altering ecosystems and biodiversity. As temperatures and weather patterns shift, the behaviour and distribution of microorganisms may change leading to a possible increase in food and feed contamination (Moretti et al., 2019; Paterson and Lima, 2017). Biological control is the most effective strategy exploited to reduce aflatoxin production, but climate changes can influence differently the behaviour of biocontrol and target agents (Zanon et al., 2023). Therefore, understanding the effectiveness of biocontrol agents in the context of climate change is crucial. This work examined, for the first time, the impact of abiotic climate change-related factors (T , a_w , and CO_2) on the growth and mycotoxin production of *A. flavus* strains, focusing on the effectiveness of a non-aflatoxigenic strain, the active agent of AF-X1, exploited as a biocontrol to reduce the aflatoxins produced in peanut.

Considering the interest in increasing peanut cultivation in North Italy and the occurrence of aflatoxin contamination in maize over the last 20 years, in the same geographic areas, this study considered two peanut varieties cultivated in Italy and two *A. flavus* strains isolated in this country from maize in 2003 (Giorni et al., 2007), and stored in an official collection, to manage this study.

The trials were carried out *in vitro* on PBM, and *in situ*, directly on peanut seeds. The high impact of the matrix effect, *in vitro*, led to the capability of *A. flavus* to produce AFB1 under dryer and warmer conditions than in previous studies with different growth media. Baazeem et al. (2021b), inoculated *A. flavus* on a 3% milled pistachio-based media, and after 10 days of incubation, no contamination was detected at $a_w \leq 0.90$ (Baazeem et al., 2021b). Whereas, we used a higher percentage of food (peanuts) in the media to obtain a substrate with nutritional characteristics as close as possible to those of peanuts, similarly to (Lazzaro et al., 2013) for maize. In particular, our PBM contained about 50% of ground peanuts and the fungus was able to produce AFB1 until $a_w = 0.85$. Similarly, the optimal T for AFB1 production *in vitro* was observed at 30°C, and such evidence is in contrast with previous studies on the same Italian aflatoxigenic strain of *A. flavus* (Camardo Leggieri et al., 2019; Giorni et al., 2011). In particular, the authors tested a T range of 15–30°C (5°C step), on a 3% maize flour-based medium, and the AFB1 optimum was observed between 20 and 25°C.

Comparing the assays carried out on PBM and peanut seeds, the highest mycotoxin production was detected *in vitro*. In particular, AFB1 produced by the aflatoxigenic strain grown alone on peanut seeds was about 50% lower compared to the content in the PBM. Nevertheless, the application of the non-aflatoxigenic strain resulted in an AFB1 reduction of 55% on PBM and over 70% on peanut seeds. These findings are in partial agreement with the study carried out on 3% milled maize agar and maize grain by Mohale et al., (2013). The authors observed that the AFB1 inhibition was slightly higher *in vitro* (up to 80%) than *in situ* where the reduction reached 77.2%. However, as suggested by Magan and Aldred (2008), fungal species and strains are in a state of flux and the changes in environmental and nutritional factors can influence their interactions. Indeed, the different foods (peanut vs. maize) and their content in the media may have played a role in the fungal interaction between our work and the one of Mohale et al. (2013).

Despite the differences caused by the media composition, the non-aflatoxigenic strain was always effective in the AFB1 control when treated with extreme conditions (high T , low a_w , high CO_2). In particular, the reduction of AFB1 slightly increased from 54% to 58% and from 71% to 76% moving from 30°C to 35°C, respectively *in vitro* and *in situ*. This is supported by the I_D scores that showed a dominance of the non-aflatoxigenic strains under the driest condition ($a_w = 0.85$) when T was the highest (35°C) and the CO_2 level increased to 1000 ppm. Similarly, Gasperini et al., (2019) investigated the effects of elevated CO_2 and T on the biocontrol using non-aflatoxigenic *A. flavus* strains in pre- and post-harvest on two maize cultivars (genetically modified, GM, and non-GM). Focusing on the post-harvest stage, which could be similar to the conditions used in this work, the biocontrol agent conserved the capability to reduce AFB1 contamination up to 71%, on GM maize, under CC conditions (37°C $\times$ 1000 ppm CO_2 and drought stress).

This efficacy under extreme climatic conditions is crucial for the field application of the biocontrol because, as stated in many studies, the increasing CO_2 will improve mycotoxin production under stress conditions (Abdelmohsen et al., 2021; Cervini et al., 2024; Garcia-Cela et al., 2021) and the drought stress may reduce the plant defence against *A. flavus* infection and aflatoxin contamination (Craufurd et al., 2006; Wotton and Strange, 1987).

Aspergillus flavus produces a wide range of secondary metabolites (Kos et al., 2022; Navale et al., 2021). In this work, when the biocontrol agent was applied, together with a general reduction in mycotoxin contamination, there was an increase in kojic acid production both *in vitro* and *in situ*. Similar results were reported by Sweany et al., (2021) who observed the greatest reads per kilobase per million mapped reads (RPKM) for the genes associated with the synthesis of kojic acid. However, kojic acid has a key role in the optical selection of contaminated products, and the increase in its production could represent an issue during the analyses to select the income products in companies. The optical method of the bright greenish-yellow fluorescence (BGYF) exploits the fluorescence of *A. flavus* under 365 nm ultraviolet (UV) (Gloria et al., 1998; Yao et al., 2023), this is related to metabolites produced by this fungus, such as Kojic acid. Indeed, as observed by Wicklow, (1999) on maize, the non-aflatoxigenic strains of *A. flavus* led to BGYF seeds, causing some “false positive” results in terms of contamination.

The AFB1 reduction obtained in the present study was in accordance with a previous study carried out on the same non-aflatoxigenic strain exploited in this work by Mauro et al., (2015). The authors observed a percentage reduction of 72% when the strain MUCL54911 was inoculated at a 1:1 ratio with an aflatoxigenic *A. flavus*, on maize kernel, under optimal growth conditions (T=31°C for 7 days). However, it is important to consider that the biocontrol agents are not applied to the harvested products, like peanut seeds or maize kernels, but it is distributed in the field at an early growth stage of crops, and the effectiveness may increase above 90%, as observed by Mauro et al., (2018).

In summary, this study has shown that the Italian non-aflatoxigenic strain of *A. flavus*, which represents the biocontrol agent of the commercial product AFX1, conserved the AFB1 reduction ability under simulated CC conditions. Aflatoxin contamination is currently a critical aspect not only in Italy but also in the whole Mediterranean region (Battilani et al., 2016). Ouadhene et al., (2024) investigated the structure and genetic variability of the *A. flavus* population in three European hotspots for aflatoxin contamination (Serbia, Greece and Spain). The study highlighted that, despite the existence of many genetic groups (Vegetative Compatibility Group, VCG), some members of the VCG IT006, which includes *A. flavus* MUCL54911, were identified.

These findings proved that this VCG is endemic in several European countries and has a high resilience to different environments, suggesting the possible effectiveness of AFX1 throughout the southern part of Europe, irrespective of climate change effects.

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CRedit:

Matteo Crosta: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Carla Cervini:** Formal analysis, Methodology, Writing – review and editing. **Angel Medina:** Conceptualization, Methodology, Resources, Supervision, Writing – review and editing. **Paola Battilani:** Conceptualization, Methodology, Resources, Supervision, Writing – review and editing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

Data availability

Data will be made available upon request.

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Figure caption

Figure 1. Production ($\mu\text{g/kg}$) of aflatoxin B1 (AFB1) (**A**) and kojic acid (KA) (**B**) by an aflatoxigenic strain of *A. flavus* grown alone or co-inoculated with a non-aflatoxigenic strain of *A. flavus*, at different T (25, 30, 35 °C), on peanut based medium. The bars indicate the mean standard error.



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Impact of simulated climate change conditions on *Aspergillus flavus* biocontrol effectiveness in peanut-based medium and peanut seeds

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