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ORIGINAL ARTICLE

Mutual effects on mycotoxin production during co-culture of ochratoxigenic and aflatoxigenic *Aspergillus* strains

Chananya Chuaysrinule[1] · Thanapoom Maneeboon[1] · Warapa Mahakarnchanakul[2]

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Abstract

Mycotoxin co-occurrence compromises the safety of food crops worldwide. Environmental factors, as well as fungal interaction, can substantially influence the infectivity of mycotoxigenic fungi and their subsequent production of multi-mycotoxin. Here, we investigated the mutual effects of the co-culture of ochratoxigenic and aflatoxigenic *Aspergillus* strains on the co-production of ochratoxin A (OTA) and aflatoxin B1 (AFB1). Single cultures of ochratoxigenic *A. carbonarius* and *A. alliaceus* grew optimally at 25 °C, whereas aflatoxigenic *A. flavus* grew optimally at 35 °C. The maximum levels of OTA and AFB1 were achieved at 25 °C, whereas mycotoxin production decreased at 35 °C. During competitive growth of the ochratoxigenic and aflatoxigenic isolates, inhibition or stimulation of mycotoxin production was dependent on the fungal strain, temperature, and the ratio of the spore concentration. *Aspergillus carbonarius* and *A. alliaceus* generally produced OTA, with similar patterns of relative OTA levels at all temperatures. AFB1 production by *A. flavus* in the presence of ochratoxigenic *Aspergillus* species was inhibited at 25 °C and stimulated at 35 °C. These results indicated that the temperature, presence of other mycotoxigenic *Aspergillus* species, and ratio of the initial spore concentration significantly contributed to the co-production of OTA and AFB1.

Keywords Mycotoxin · Aflatoxin · Ochratoxin A · *Aspergillus* · Co-culture · Fungal interaction · Co-occurrence

Abbreviations

AFB1 Aflatoxin B1
OTA Ochratoxin A

Introduction

Mycotoxins are low-molecular weight secondary metabolites of fungal genera such as *Aspergillus*, *Fusarium*, and *Penicillium*. A survey of mycotoxins in grains intended for human and animal consumption found that the global frequency of mycotoxins was in the range of 60–80% (Eskola et al. 2019). Aflatoxins are the most toxic mycotoxins with potent carcinogenic and mutagenic activities. Aflatoxins are produced mainly by *Aspergillus* section *Flavi*, among which *A. flavus* and *A. parasiticus* are the major producers. Aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2) are the main naturally occurring aflatoxins, of which, AFB1 is the most common and toxic aflatoxins (Cervino et al. 2007). Ochratoxin A (OTA) is a nephrotoxic mycotoxin produced by several species of *Aspergillus* and *Penicillium*. In tropical regions, OTA contamination of food and feed products is typically caused by *Aspergillus* section *Nigri*, including *A. niger* and *A. carbonarius* (Alvindia and de Guzman 2016); section *Circumdati*, including *A. ochraceus* and *A. westerdijkiae* (Gil-Serna et al. 2015); and the *A. alliaceus* clade in the section *Flavi* (Frisvad et al. 2019).

Mycotoxigenic fungi present in food commodities produced in tropical regions can be exposed to higher temperatures, especially during the sun-drying process. For example, chili is traditionally dried in direct sunlight to reduce its moisture content from approximately 80 to 10% (Fudholi

✉ Warapa Mahakarnchanakul
fagiwpm@ku.ac.th

Chananya Chuaysrinule
rdicnc@ku.ac.th

Thanapoom Maneeboon
thanapoom.m@ku.th

[1] Scientific Equipment and Research Division, Kasetsart University Research and Development Institute (KURDI), Kasetsart University, Bangkok 10900, Thailand

[2] Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand

et al. 2013), which requires 7–20 days, depending upon the quality of sunlight, temperature, and humidity (Toontom et al. 2016). Changes in the proportion of mycotoxigenic fungi can occur during the drying process of agricultural products. As reported by Costa et al. (2020), *A. flavus* was initially present in a low amount (14%) in fresh chili fruits, whereas the populations of *A. niger* and *A. flavus* after drying increased by 50% and 25%, respectively. Thus, dried chili products are frequently contaminated with OTA, aflatoxins, or both, particularly those produced in hot and humid conditions (Santos et al. 2011; Yogendrarajah et al. 2014; Ali et al. 2015; Gambacorta et al. 2018).

The interactive toxicity effect of co-exposure to multi-mycotoxin can be antagonistic, additive, or synergistic effects (Joshi et al. 2022). For example, OTA could increase the mutagenic effect of AFB1 in cases of co-occurrence in the same crop (Sedmíková et al. 2001). Contamination of multi-mycotoxin in food and feed has been recognized as an emerging risk with regard to human and animal health (Palumbo et al. 2020). Thus, an effective and sustainable mycotoxin mitigation tool could enhance global food safety and security, which are critical factors in achieving the sustainable development goals (SDGs) adopted by all member states of the United Nations (Ortega-Beltran and Bandyopadhyay 2021).

Change in climate impacts significantly the productivity and quality of crops, the fungal colonization pattern, and the excretion of various fungal metabolites including mycotoxins. Currently, concurrent heat and drought conditions related to actual climate change are expected to have an impact on the increased levels of aflatoxins in maize (Chhaya et al. 2022). Numerous studies on fungal interactions influenced by climate change have focused on the co-cultivation of aflatoxigenic *A. flavus* and mycotoxigenic *Fusarium* species, particularly in pre-harvest maize (Stagnati et al. 2020; Giorni et al. 2019; Camardo Leggieri et al. 2019). Although several reports have investigated the competitiveness of *A. flavus* with ochratoxigenic *A. niger* (Barberis et al. 2012) and *A. carbonarius* (Barberis et al. 2014), there has been no report on the influence of temperature on the growth and mycotoxin production of co-cultures between aflatoxigenic and ochratoxigenic *Aspergillus* species.

To fill this gap in our knowledge, we report here the in vitro effects of temperature on the growth and production of OTA and AFB1 in the co-culture of ochratoxigenic *A. carbonarius* or *A. alliaceus* with aflatoxigenic *A. flavus*. The relationships were also investigated between temperature, the ratio of initial spore concentration and incubation time on the growth and production of mycotoxins by ochratoxigenic and aflatoxigenic *Aspergillus* strains under co-cultivation.

Materials and methods

Fungal isolates

Ochratoxigenic *A. carbonarius* CH112 and *A. alliaceus* CH132 and aflatoxigenic *A. flavus* CH141 previously isolated from dried chili were used. Potato dextrose agar cultures were maintained at room temperature. Spores were collected by scraping the mycelia from 7-day cultures using 10 ml of sterile 0.01% (v/v) Tween 20 and then counted using a hemocytometer. The spore concentrations were adjusted to obtain the desired concentration using sterile 0.01% (v/v) Tween 20.

Inoculation, incubation, and fungal competition

Yeast extract sucrose (YES) medium, which is a conducive medium for mycotoxin production by *A. carbonarius*, *A. alliaceus*, and *A. flavus* (Bayman et al. 2002; Fountain et al. 2016), was used to evaluate the fungal growth and production of OTA and AFB1. This medium is used for screening the mycotoxin producing ability and investigating the effect of environmental factors on growth, mycotoxin production and gene expression of mycotoxigenic *Aspergillus* species in previous studies (Singh et al. 2020; Wang et al. 2020; Abdel-Hadi et al. 2021). Single cultures of each isolate were prepared using 20 µl of a spore suspension containing 10^2 or 10^4 spores/ml that were pipetted onto the center of the YES plate. Then, the inoculated plates were sealed and incubated at 25 °C or 35 °C in the dark for 21 days. In the co-culture experiment, different spore mixtures of ochratoxigenic (*A. carbonarius* or *A. alliaceus*) and aflatoxigenic strains at ratios of 10^2:0, 10^4:0, 10^2:10^2, 10^4:10^4, 10^2:10^4, 10^4:10^2, 0:10^2, and 0:10^4 spores/ml were made by mixing the required volume of spore suspension in a test tube to obtain the different spore concentration ratios. A 20-µl sample of each mixed spore suspension was inoculated onto the center of the YES plate, which was incubated as described above. All experiments were performed in triplicate and repeated twice.

Growth assay

Fungal growth was assessed by measuring the diameter of a colony in two directions at right angles. Measurements were performed after 2, 3, 5, 7, 14, and 21 days of incubation. In the co-culture experiment, the fungal growth rate was analyzed by plotting the colony diameter (mm) against incubation time (days) and then calculating the slope of the regression line using the Microsoft Excel software package.

Mycotoxin analysis

The production of OTA and AFB1 was analyzed after 7, 14, and 21 days of incubation. Five agar plugs from the YES plates were randomly cut from different positions within a colony, weighed, extracted with 2 ml of methanol, and sonicated for 30 min. The mixture was passed through a 0.45-µm syringe membrane filter before injection into an HPLC equipped with a fluorescence detector (2690/95, Waters Corporation, MA, USA). The HPLC conditions used to analyze each mycotoxin were implemented according to a published method (Chuaysrinule et al. 2020a). HPLC analysis was performed using a Symmetry C18 column (5 µm, 3.9 × 150 mm) (Waters Corporation, MA, USA) at 35 °C and a flow rate of 1 ml/min. The mobile phase used to analyze OTA was 6% (w/v) acetic acid in the water, acetonitrile, and methanol (45:35:20). OTA was detected using the excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 350 nm and 470 nm, respectively. To analyze AFB1, post-column derivatization was performed using a photochemical reactor for enhanced detection (Aura Industries Inc., CA, USA). The mobile phase comprised water, acetonitrile, and methanol (60:15:25). AFB1 was detected at λ_{ex} 365 nm and λ_{em} 445 nm. The detection and quantification limits for OTA in

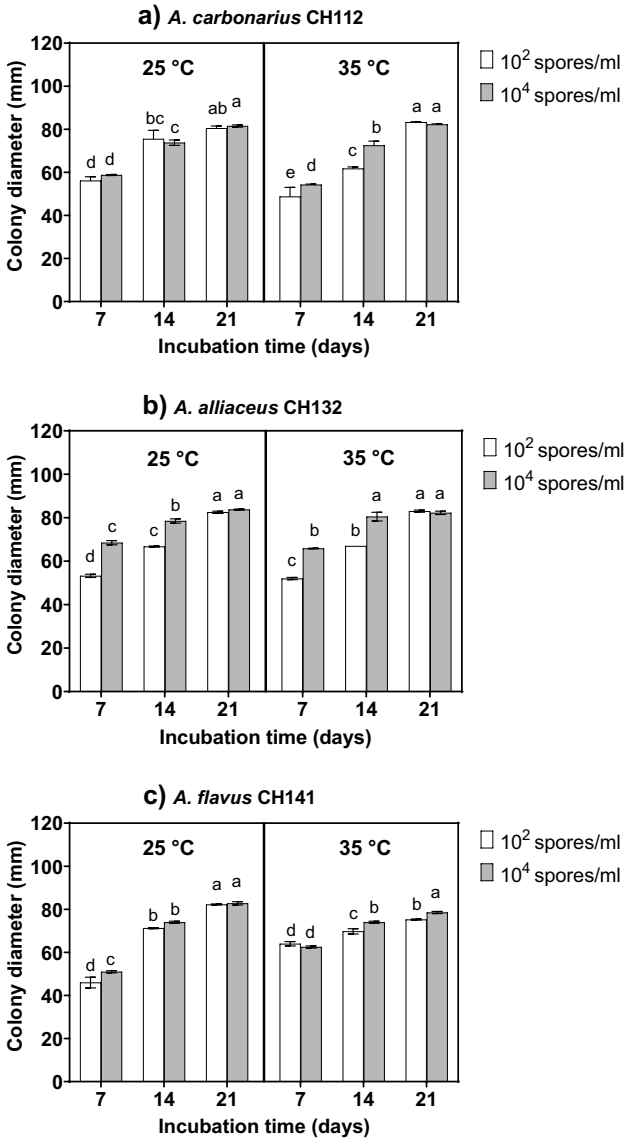

Fig. 1 Effects of temperature, spore concentration, and incubation time on radial growth of ochratoxigenic and aflatoxigenic *Aspergillus* strains individually cultured in YES medium. Data are expressed as mean ± SEM. Different letters indicate a significant ($P < 0.05$) difference based on Fisher's least significant difference

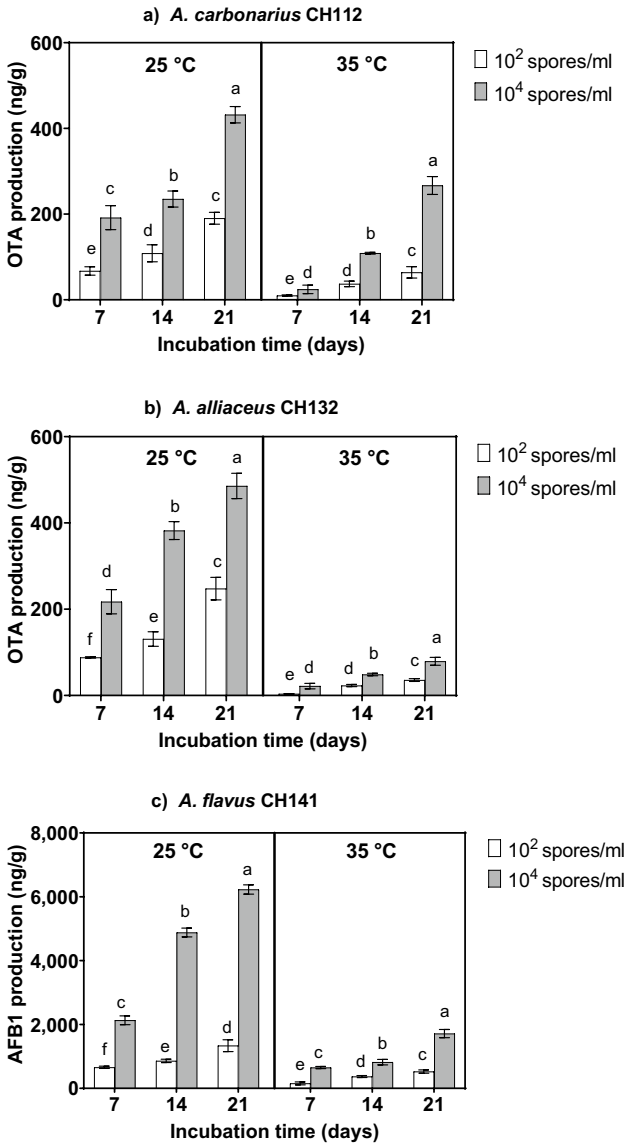

Fig. 2 Effects of temperature, spore concentration, and incubation time on the production of OTA by ochratoxigenic *Aspergillus* strains and AFB1 by *A. flavus* individually cultured in YES medium. Data are expressed as mean ± SEM. The different letters indicate a significant ($P < 0.05$) difference based on Fisher's least significant difference

Table 1 Effects of ochratoxigenic *Aspergillus* species, temperature, ratio of spore concentration, and incubation time on overall growth, relative OTA levels, and relative AFB1 levels of co-cultures of two ochratoxigenic species and aflatoxigenic *A. flavus*

Source	df	Fungal growth rate		Overall relative OTA production		Overall relative AFB1 production	
		MS	*F* value[a]	MS	*F* value	MS	*F* value
Ochratoxigenic species (*S*)	1	17.59	532.35**	6.60	2083.45**	0.05	13.05**
Temperature (*T*)	1	11.40	345.00**	0.80	251.03**	3.92	1101.48**
Spore-concentration ratio (*I*)	3	13.94	422.06**	7.60	2398.73**	5.26	1478.58**
Time (*t*)	2	109.41	3311.82**	0.22	69.31**	0.11	31.45**
$S\times T$	1	5.75	174.02**	0.00	1.39^{NS}	2.42	681.03**
$S\times I$	3	0.30	9.23**	2.09	660.98**	0.52	145.19**
$S\times t$	2	2.30	69.68**	0.38	118.99**	0.02	5.72**
$T\times I$	3	3.58	108.51**	0.44	139.07**	0.63	177.43**
$T\times t$	2	2.28	69.15**	0.33	102.93**	0.83	234.30**
$I\times t$	6	7.82	236.63**	1.29	405.58**	0.17	47.36**
$S\times T\times I$	3	0.46	13.93**	2.19	690.75**	0.60	169.60**
$S\times T\times t$	2	0.69	20.80**	1.27	399.57**	0.41	114.48**
$S\times I\times t$	6	0.42	12.65**	1.26	396.30**	0.09	25.03**
$T\times I\times t$	6	1.11	33.66**	0.59	186.08**	0.22	60.88**
$S\times T\times I\times t$	6	0.59	17.95**	0.44	137.46*	0.19	53.70**

[a]**Significant, $P<0.01$; *significant, $P<0.05$; NS, not significant, $P>0.05$

uninoculated media were 0.19 ng/g and 0.25 ng/g, respectively, and were 0.11 ng/g and 0.25 ng/g, respectively, for AFB1. The average recovery of both mycotoxins was in the range 70–110%, with a relative standard deviation of < 20%.

Data analysis

The levels of OTA and AFB1 produced in the co-culture experiments were normalized to those of the culture containing only each ochratoxigenic or aflatoxigenic strain at the same temperature, spore concentration, and incubation time (Furukawa et al. 2016). Normalized data were expressed as the relative mycotoxin level. Analysis of variance (ANOVA) and Pearson's correlation analysis were performed to evaluate the significance of differences in growth rate, mycotoxin production, and relative mycotoxin levels. Fisher's least significant difference was used to compare differences in mean values with $P<0.05$ indicating a significant difference. Statistical analyses were performed using the MATLAB R2021a (MathWorks, Inc, Natick, MA, USA).

Results and discussion

Fungal growth and production of OTA and AFB1 in single cultures of ochratoxigenic and aflatoxigenic *Aspergillus* strains

Figure 1 shows the influence of temperature and initial spore concentration on the colony diameters of *A. carbonarius*, *A. alliaceus*, and *A. flavus* grown on YES medium. Figure 2 shows the OTA and AFB1 levels produced by these fungi cultured on the same medium at 25 °C and 35 °C. The results showed that the fungal growth and production of OTA and AFB1 were significantly affected by the temperature and initial spore concentration.

The highest growth and OTA production by *A. carbonarius* were achieved at 25 °C. Previously, maximum growth and OTA production by *A. carbonarius* were reported in the temperatures ranges 20–35 °C and 15–30 °C, respectively (Alborch et al. 2011; Chuaysrinule et al. 2020b; Mutlu-Ingok and Karbancioglu-Guler 2014). The apparent low frequencies of *A. alliaceus* in food and feed commodities are reflected in the few published reports on the effects of physiological factors on fungal growth and production of OTA (Bayman et al. 2002). The present findings showed that OTA was optimally produced at 25 °C and that there was no significant difference between the growth of *A. alliaceus* at 25 °C or 35 °C. Similar results were reported where the most abundant growth in culture media of *A. alliaceus* isolated from animal feed occurred at 25 °C (Bouti et al. 2020); however, that study did not examine OTA production by the tested fungal isolates.

For *A. flavus*, we found the highest fungal growth and AFB1 levels produced by aflatoxigenic *A. flavus* were achieved at 25–35 °C and 35 °C, respectively. These findings agreed with other previous reports that found that the optimum temperatures for aflatoxin production were in a narrower range than those for fungal growth (Aldars-García et al. 2018; Norlia et al. 2020).

We found that an increase in the initial spore concentration contributed to a high growth rate and increased the

amounts of OTA and AFB1 produced by all tested *Aspergillus* strains. These results correlated with other finding where in a glucose minimal salt medium, the mycelial dry weight and AFB1 production increased when the initial spore concentration increased from 10^4 to 10^6 spores/ml (Yan et al. 2012). In a similar study, Li et al. (2017) showed that the OTA production by *A. ochraceus* grown in potato dextrose broth increased when the initial spore concentration was increased from 10 to 10^6 spores/ml. It is possible that a high initial spore concentration promotes rapid mycelial growth and subsequent mycotoxin production.

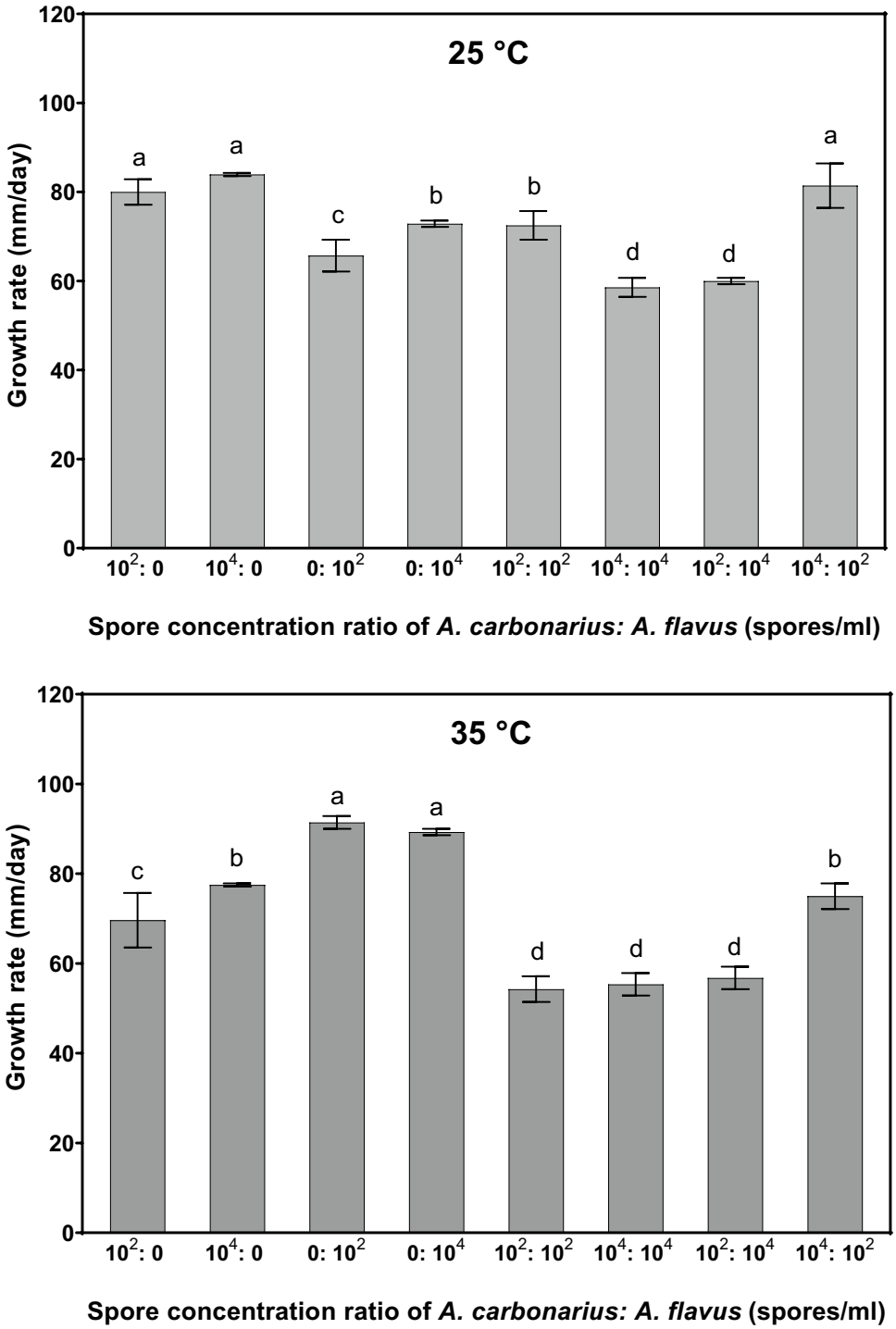

Fig. 3 Growth rates of co-culture between *A. carbonarius* and *A. flavus* in YES medium at different ratios of spore concentrations. Data are expressed as mean ± SEM. Different letters indicate a significant ($P < 0.05$) difference based on Fisher's least significant difference

Fungal growth and production of OTA and AFB1 in co-cultures of ochratoxigenic and aflatoxigenic *Aspergillus* strains

The ANOVA analyses of the growth rates and production of OTA and AFB1 are shown in Table 1. Ochratoxigenic species, temperature, spore-concentration ratio, and incubation time, as well as their interactions, highly significantly influenced growth rates ($P<0.01$). All single and interactive factors, other than the interaction between the ochratoxigenic *Aspergillus* species and temperature ($P>0.05$), showed a significant influence on the relative levels of OTA. Furthermore, the changes in AFB1 production by *A. flavus* in the presence of ochratoxigenic species were significantly affected by all single factors and their interactions ($P<0.01$).

Pearson correlation analysis revealed a significant, positive correlation between growth rates and relative OTA level ($r=0.226$, $P<0.01$). Temperature was significantly

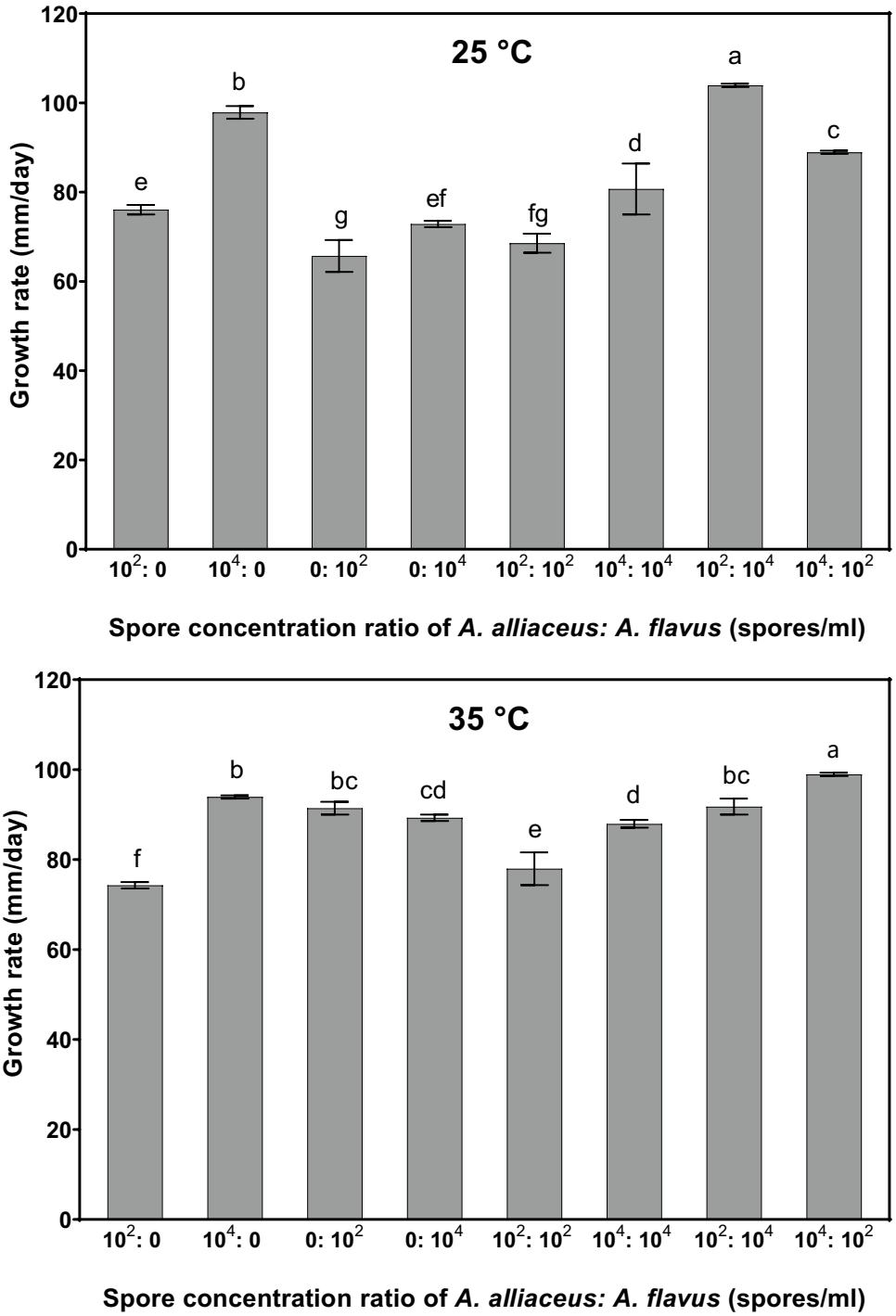

Fig. 4 Growth rates of co-culture between *A. alliaceus* and *A. flavus* in YES medium at different ratios of spore concentrations. Data are expressed as mean ± SEM. Different letter indicate a significant ($P<0.05$) difference based on Fisher's least significant difference

associated with an increase in the relative AFB1 level ($r = 0.337$, $P < 0.01$) as well as having a significant, negative correlation with fungal growth rate ($r = 0.337$, $P < 0.01$). Furthermore, relative OTA level positively affected relative AFB1 level ($r = 0.241$, $P < 0.05$). However, there was significant correlation neither between the temperature and the relative OTA level nor between the growth rate and the relative AFB1 level ($P > 0.05$).

The two ochratoxigenic *Aspergillus* species used in this study displayed different growth patterns in co-culture with *A. flavus*. A significant reduction in growth rates ($P < 0.05$) was achieved in co-cultures of *A. carbonarius* and *A. flavus* at both the investigated temperatures (Fig. 3). In contrast, a significant increase in the growth rate ($P < 0.05$) was observed when *A. alliaceus* was cultured together with *A. flavus* at 25 °C and a spore concentration ratio of 10^2:10^4 spores/ml (Fig. 4). Furthermore, almost all combinations of co-culture of *A. alliaceus* and *A. flavus* did not produce a significant decrease ($P > 0.05$) in the fungal growth rate, particularly at 35 °C. The adverse effects of co-culture on fungal growth have been observed in several combinations of aflatoxigenic *A. flavus* strains (Wicklow et al. 2003) as well as those of ochratoxigenic *P. verrucosum* and *P. nordicum* against various species of *Aspergillus*, *Penicillium*, and *Trichoderma* (Vankudoth et al. 2016) and among aflatoxigenic *A. flavus* and fumonisin-producing *F. verticillioides* (Yan et al. 2021). Daly et al. (2017) suggested that the decrease in fungal growth in the co-culture indicated antagonism between the two fungal species or strains.

Figure 5 shows the relative changes in OTA and AFB1 production in the co-culture of the ochratoxigenic and aflatoxigenic *Aspergillus* strains. OTA production was significantly inhibited ($P < 0.05$) when an equal or higher proportion of aflatoxigenic *A. flavus* was cultured with both ochratoxigenic species at all incubation temperatures. In particular, OTA production by *A. carbonarius* (*Aspergillus* section *Nigri*) was undetectable (relative OTA, − 1.00) in co-culture with a spore concentration ratio of 10^2:10^2 spores/ml. For *A. alliaceus*, complete OTA inhibition occurred at 35 °C on day 7 in cultures initiated with an equal proportion of spores and on days 7–21 in the culture containing a higher proportion of *A. flavus*. In line with our findings, Kogkaki et al. (2015) reported that OTA production in the dual culture between *A. carbonarius* strains and other grape-related *Aspergillus* section *Nigri* species was most inhibited and less stimulated depending on the fungal competitors and environmental conditions. However, our results were inconsistent with another study reporting mycotoxin production in an interactive cultures of *A. flavus* and *A. niger* aggregate strains at 28 °C (Barberis et al. 2012), where the authors observed the stimulation of OTA, whereas the AFB1 production was reduced in all inoculum sizes and incubation times of co-culture compared to those produced in the single culture.

In the present study, AFB1 production by *A. flavus* was significantly influenced by the presence of ochratoxigenic species ($P < 0.01$). Compared with the single culture of *A. flavus* at 25 °C, AFB1 production in the co-culture with the two ochratoxigenic strains was generally reduced in almost all mixed cultures. Furthermore, our results showed that the temperature significantly influenced the relative aflatoxin levels ($P < 0.01$) in co-cultures of *A. alliaceus* and *A.*

Co-culture	Spore concentration ratio	25 °C						35 °C					
		Relative OTA level			Relative AFB1 level			Relative OTA level			Relative AFB1 level		
		7 D	14 D	21 D	7 D	14 D	21 D	7 D	14 D	21 D	7 D	14 D	21 D
A.c. (OTA+) + *A.f.* (AF+)	10^2:10^2	-1.00	-0.95	-1.00	-0.80	-0.75	-0.96	-1.00	-1.00	-1.00	-0.71	-0.90	-0.99
	10^4:10^4	-0.76	-0.91	-0.91	-0.89	-0.91	-0.90	1.70	-0.65	-0.83	-0.79	-0.54	-0.83
	10^2:10^4	-0.45	-0.74	-0.60	0.29	-0.28	0.15	-0.38	-0.24	-0.24	0.13	0.46	-0.26
	10^4:10^2	-0.26	2.42	0.66	-0.13	-0.23	-0.33	0.01	0.69	0.33	-0.27	-0.13	-0.06
A.a. (OTA+) + *A.f.* (AF+)	10^2:10^2	-0.50	-0.30	-0.80	-0.97	-0.96	-0.93	-1.00	-0.48	-0.82	-1.00	-0.88	-0.77
	10^4:10^4	-0.48	-0.29	-0.59	-0.83	-0.80	-0.41	-1.00	-0.85	-0.84	-0.44	-0.04	-0.69
	10^2:10^4	-0.90	-0.90	-0.83	-0.83	-0.88	-0.81	-1.00	-1.00	-1.00	0.66	0.73	-0.23
	10^4:10^2	0.61	1.11	0.79	-0.87	-0.89	-0.10	-0.32	0.08	0.37	0.60	0.00	-0.15

A.c. (OTA+) : ochratoxigenic *A. carbonarius*
A.a. (OTA+) : ochratoxigenic *A. alliaceus*
A.f. (AF+) : aflatoxigenic *A. flavus*

-1 0 2.5

Fig. 5 Heat-map analysis of relative levels of OTA and AFB1 in co-cultures of ochratoxigenic and aflatoxigenic *Aspergillus* strains in YES medium at different temperatures, ratios of the initial spore concentrations, and incubation times

flavus, which are members of the *Aspergillus* section *Flavi*. As the temperature increased to 35 °C, stimulation of AFB1 production occurred during the first 14 days in almost all co-cultures with different proportions of spores of each species. Thus, the relative AFB1 levels gradually reduced after 21 days of incubation. It is known that the production of mycotoxins in a fungal community serves as a defense mechanism against a fungal competitor to maintain colonization of the substrate under stress conditions caused by an increased temperature (Magan et al. 2010). A similar finding was reported in the co-culture of aflatoxigenic *Aspergillus* belonging to section *Flavi* (Ching'anda et al. 2021); they reported a twofold increase in aflatoxin production in co-cultures of *A. flavus* with *A. parasiticus* or *A. aflatoxiformans* with an increase in temperature from 25 to 30 °C. In general, 35 °C is a typical average ambient temperature during the sun-drying process of crops (Seetapong et al. 2017). The present findings were supported by the results of Valente et al. (2020) who reported that the largest population of *A. flavus* and the highest level of aflatoxins were detected in hazelnuts dried at 35 °C for 33 h after storage for 14 days. Those authors recommended shorter drying time and higher drying temperature (45 °C) to limit fungal growth and aflatoxin contamination of hazelnuts.

To our knowledge, few studies have focused on the interaction of co-cultures of ochratoxigenic and aflatoxigenic strains. To explore the real effect of abiotic and biotic factors on the complex interaction between two different mycotoxigenic species, the experiment was first conducted on the YES medium. The present work revealed that OTA and AFB1 production in the co-culture was inhibited or stimulated, depending on the ochratoxigenic species, ratio of spore concentration, and temperature. Our preliminary findings should be useful for understanding the complex fungal interaction and resulting co-production of OTA and AFB1. However, there were two limitations in our study. The first one was that we did not investigate the fungal interaction on food matrices. Since the chemical composition and microstructure of the food are known to exert a significant effect on fungal growth and mycotoxin production (Marín et al. 2021), the fungal behavior obtained from the present study needs to be validated on food matrices. The second limitation was that only one strain of each *Aspergillus* species was used, which did not allow for evaluating the variability between fungal strains. A further study should use large strain collections of different sources to assess the strain variability during the co-culture.

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Author contribution CC: performed experiments and analyzed the data. TM: analyzed the data and drafted the manuscript. WM: designed study, analyzed data, and edited the manuscript.

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Availability of data and material (data transparency) Not applicable.

Code availability (software application or custom code) Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

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