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Interactive Inhibition of Aflatoxigenic *Aspergillus flavus* and Ochratoxigenic *Aspergillus carbonarius* by *Aspergillus oryzae* under Fluctuating Temperatures

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Abstract: This study aimed to evaluate the effectiveness of *A. oryzae* in inhibiting aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) production by *A. flavus* and *A. carbonarius*, respectively, under shifting temperatures. *A. oryzae* was tested on different agar, namely coconut cream agar (CCA) and chili-based agar to figure out the variation in the effectiveness of *A. oryzae* on the most appropriate medium for *A. flavus* and *A. carbonarius* to produce mycotoxin and under natural condition where they are predominantly found. On CCA, the temperatures applied were 20, 30, 35, 40, 20/30, 20/35, and 20/40 °C, while on chili-based agar, the temperatures imposed were 20, 40, and 20/40 °C, at varied water activity of 0.92 and 0.97a_w. The findings indicated that *A. oryzae* was much more effective in inhibiting the growth of *A. flavus* rather than *A. carbonarius*, yet it was able to inhibit higher OTA concentration than AFB_1 at fluctuating temperatures on CCA as the most appropriate medium for *A. flavus* and *A. carbonarius*. *A. oryzae* effectively inhibited AFB_1 and OTA at static temperature of 20 °C and water activity of 0.97a_w on chili-based agar. Under fluctuating temperatures (20/40 °C), *A. oryzae* was also able to control mycotoxin, particularly OTA at high water activity (0.97a_w).

Keywords: aflatoxin B_1; ochratoxin A; biocontrol; fluctuating temperatures; fungal interaction

1. Introduction

Mycotoxins are low-molecular-weight natural products produced as secondary metabolites by filamentous fungi, and they have toxic effects on humans and animals [1]. Aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) are primary mycotoxins studied as they are found mainly in crops. Aflatoxins mostly contaminate cereals, oilseeds, nuts, dried fruits, spices, and beans, while OTA is found in grapes and coffee beans [2,3]. Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *Aspergillus flavus* and *A. parasiticus*. Meanwhile, OTA is predominantly produced by *A. ochraceus*, *A. niger*, *A. carbonarius*, *Penicillium verrucosum*, and *P. nordicum* [4–8].

Chili is one of the ubiquitous crops known for its mycotoxin contamination. Previous studies have demonstrated the occurrence of aflatoxins and OTA in chili from several countries, such as Indonesia, Malaysia, Pakistan, Bangladesh, Qatar, Saudi Arabia, and Thailand. In addition, mostly isolated fungi from chili include *A. parasiticus*, *A. flavus*, *A. nomius*, and *A. niger* [9–15].

Among fungi that have been isolated from chili, *A. oryzae* is also found as a nontoxigenic fungi [16,17]. The strains of this species have various mutations within aflatoxin biosynthetic gene clusters, resulting in their inability to produce aflatoxins; in particular, the aflR gene is absent or significantly different in some *A. oryzae* compared to *A. flavus*.

Both the species are typically found in cereal, soil, or koji fermentations used for miso, sake, and other Japanese and Korean fermented products [18,19]. *A. oryzae* was used as a biocontrol agent for controlling mycotoxin producing fungi. In addition, it is known as a Generally Recognized as Safe (GRAS) microorganism [20,21]. Previous studies showed that *A. oryzae* reduced aflatoxin [22–24]. However, there are few studies on the optimum environmental conditions for *A. oryzae* to reduce aflatoxin and OTA production.

Detoxifications of mycotoxins can be performed by physical, chemical, and biological methods. However, the biological method remains preferable due to safety issues, possible losses in nutritional contents, limited efficacy, and cost implications in physical and chemical controls [25]. Several fungi have succeeded in controlling AFB$_1$ and OTA contamination, such as atoxigenic *A. flavus* and *A. parasiticus*, *Trichoderma* sp., *Phoma* sp., *Rhizopus* sp., *Sporotrichum* sp., *Alternaria* sp., and *A. pullulans* [26–28]. The environmental factors, including temperature and water activity, also affect their effectiveness. For instance, the maximum activity of protease produced by *Trichoderma harzianum* to control toxigenic fungi was obtained at a temperature of 40 °C [29]. *Debaryomyces hansenii* could stimulate aflatoxins production by *A. parasiticus* at $0.99a_w$, whereas a significant reduction of aflatoxins was observed at $0.92a_w$ [30].

Although fungal competition have been intensively studied, most of them were carried out under static temperatures [31–33]. Meanwhile, fluctuating temperatures may occur in the environment due to climatic changes, and it is able to increase mycotoxin formations, such as deoxynivalenol and zearalenone by *Fusarium graminearum* [34], aflatoxin by *A. parasiticus* [35,36], and OTA by *A. ochraceus* [37]. In this study, we aimed to understand interaction of fungi isolated from chili and study the effectiveness of *A. oryzae* to inhibit AFB$_1$ and OTA under fluctuating temperatures.

2. Materials and Methods

2.1. Fungal Strains

There were three fungal strains used in this study, *Aspergillus flavus* strain CH141, *A. carbonarius* strain CH112, and *A. oryzae* strain CH084. They were isolated from chili in Thailand and stored as culture collections of Kasetsart University Research and Development Institute (Bangkok, Thailand). All the isolates of *A. flavus* and *A. carbonarius* have been proven to produce AFB$_1$ and OTA, respectively, and *A. oryzae* was proven to produce neither of them. The analysis was conducted using thin-layer chromatography (TLC) coupled with densitometry following extraction with the protocol described by Chuaysrinule, Maneeboon, Roopkham and Mahakarnchanakul [15].

2.2. Mycological Media

Coconut cream agar (CCA) was used in this study to analyse mycotoxin production at different temperatures merely through UV light (366 nm). Additionally for *A. flavus*, it was also a suitable medium for *A. carbonarius* in producing OTA, similar to other prominent synthetic media such as potato dextrose agar (data not shown). CCA was prepared by mixing coconut cream and water with a ratio of 50:50 prior to the addition of agar 1.5% and sterilization using autoclave at 121 °C for 15 min [38].

Meanwhile, chili powder agar was used to provide the fungi with similar living conditions as in chili. Furthermore, it was preferred rather than real chili due to its efficiency in adjusting the moisture content and controlling the desired conditions. Chili powder agar 2% was made with 20 g of chili powder, 20 g of agar and 1 L of distilled water, as detailed by Marín, et al. [39]. The medium was modified with 260.95 and 134.58 g of glycerol per liter of medium to obtain 0.92 and 0.97 a_w, respectively. The values 0.92 and 0.97 a_w were within the range of optimum condition for AFB$_1$ and OTA production; moreover, 0.97 a_w was the highest water activity that could be achieved during trials in obtaining desired a_w levels. All autoclaved media were poured in 9-cm petri dishes, and final a_w levels were measured after solidification. The water activity of all media was determined using a water activity meter (AquaLab 4TE, Decagon Devices, Pullman, Washington, DC, USA).

2.3. Inoculum Preparation

Initially, each strain was inoculated on potato dextrose agar (PDA) and incubated at 25 °C for 7 days. A spore suspension was made by adding 0.1% (*v/v*) tween 20 in distilled water. The spore was harvested by addition of 5 mL of suspension previously prepared suspension. Subsequently, the spore suspension was counted using a hemocytometer and diluted to achieve a spore concentration of 10^5 spores/mL. In mixed culture experiments, a spore suspension of *A. flavus* or *A. carbonarius* was mixed with an equal volume of spore suspension of *A. oryzae*. In single cultures, spore suspension of each *A. flavus*, *A. carbonarius*, and *A. oryzae* was mixed with an equal volume of sterilized water.

2.4. Inoculation and Incubation

On CCA, 20-µL of spore suspension of single and mixed cultures, prepared as previously reported, were inoculated on the center of agar to achieve the final concentration of 10^3 spores/mL. Cultures inoculated on CCA were incubated at the static temperatures of 20, 30, 35, and 40 °C for 6 days as control, and at three levels of fluctuating temperatures, 20/30, 20/35, and 20/40 °C for 6 days in total. Under fluctuating temperatures, incubation times were 3 days at each initial and final temperature to give the same condition on the mycotoxin production. Six days were chosen because this incubation was generally sufficient for *A. flavus* to achieve significant AFB$_1$ accumulation [40]. The fluctuating temperatures were made manually by changing the temperature from low to high temperature after 3 days incubation at a low temperature.

On chili-based agar, a dual culture technique was performed. The inoculation procedure on CCA and chili-based agar was differentiated because the mycelium color of the fungi widely varied on CCA, allowing the influence of one fungus on mycelium growth to be clearly observed. On the other hand, fungi on chili-based agar have similar mycelium color that might interfere in identifying the fungus effect on mycelium growth if mixed cultures technique was applied. Dual culture was carried out by inoculating 10 µL of spore suspension of each fungal species on the medium on the opposite site at a distance of 3 cm between inoculums. The single culture was performed by inoculating only spore suspension of one isolate in a similar manner. Petri plates with chili-based agar on same water activity were stored together in polyethylene plastics and incubated at (1) static temperature of 20 and 40 °C for 7 days at each temperature as controls and at (2) fluctuating temperatures from 20 °C to 40 °C with the interval times of 15 h and 9 h at each temperature, respectively, to simulate day and night time. The cycling temperature was imposed for 4 days in total, but the fluctuating temperatures were performed after pre-incubation at a static temperature of 20 °C for 3 days. The temperatures of 20, 30, 35, and 40 °C were chosen as they are within the temperature range in Thailand [41].

2.5. Growth Assessment

The growth of fungi on CCA was recorded by measuring the diameter of mycelia in two directions at right angles to each other after 6 days of incubation. The diameter of a colony (cm) was divided by total incubation time (days) to obtain fungal growth rates (cm/day). The same approach to measure the growth was also conducted for cultures on chili-based agar, but the assessment was performed daily.

2.6. AFB$_1$ and OTA Analysis

The fungal colony grown on CCA was extracted and quantified for AFB$_1$ and OTA concentrations by thin-layer chromatography (TLC) coupled with a densitometer [42]. Five agar plugs of CCA were extracted by using 1 mL of methanol and sonicated for 60 min. Following sonication, 10 µL of supernatant in samples and 5 concentrations of AFB$_1$ and OTA standards (5, 10, 15, 20, 30 ng/mL) were spotted in thin-layer chromatography silica gel 60 plates (Merck KGaA, Darmstadt, Germany). Those standards were prepared to construct the calibration curve. The plates were developed in the mobile phase consisting of chloroform/acetone (9:1, *v/v*) for AFB$_1$ analysis and toluene/ethyl acetate/formic acid

(6:3:1, $v/v/v$) for OTA analysis with a migration distance of 8 cm. They were then left to air-dry for 5 min and scanned using a densitometer TLC scanner 3 (Camag, Muttenz, Switzerland) at the wavelength of 366 nm and 333 nm for AFB$_1$ and OTA detection, respectively. Limit of detection (LOD) and limit of quantification (LOQ) were 0.61 and 0.91 ng/spot for AFB$_1$, 3.14 and 3.39 ng/spot for OTA, respectively.

Mycotoxins from cultures on chili-based agar was assessed with different methods because the mycotoxin on chili-based agar was produced in low concentrations. Cultures cut from chili-based agar were weighted and then extracted by adding 10 mL of methanol and sonication for 60 min. The extract was filtered with a 0.45-µm syringe filter and stored in a 2-mL amber vial. According to method 991.31 in AOAC [43], reverse-phase HPLC was applied and combined with a fluorescent detector. Sample eluent was injected into HPLC (2690/95, Water Corporation, Milford, MA, USA) and a symmetry® C18 column (5 µm, 3.9 × 150 mm) (Waters Corporation) was employed. For AFB$_1$ detection, water/acetonitrile/methanol (60:15:25, $v/v/v$) was used as mobile phase at a flow rate of 1 mL/min. Column temperature was set at 40 °C. The fluorescence detection was operated using a wavelength of 365 nm and 445 nm for excitation and emission wavelengths, respectively. The post-column derivatization was conducted with a photochemical reactor for enhanced aflatoxin detection (PHRED) (Aura Industries, San Diego, CA, USA). For OTA analysis, the mobile phase was 6% (v/v) acetic acid/acetonitrile/methanol (45:35:20, $v/v/v$) at a flow rate of 1 mL/min and column temperature was 35 °C. The excitation and emission wavelengths were 350 nm and 470 nm, respectively. LOD and LOQ obtained were 0.18 and 0.31 ng/g for AFB$_1$, 0.23 and 0.36 ng/g for OTA, respectively. The recovery ranges for AFB$_1$ and OTA were 81.74–99.66% and 82.35–109.25%, respectively.

2.7. Statistical Analysis

One-way analysis of variance was performed to assess significant differences between mean values, then T-Tests and Duncan Multiple Range Test with p-value < 0.05 were applied to determine the mean differences. The analysis was carried out using Statistical Package for the Social Science (SPSS) ver. 20 program (IBM Corporation, Armonk, NY, USA). All mycotoxin production data were converted by $y = \ln(x)$ before ANOVA to homogenize the variance [44]. Experiments were conducted two times with three replications.

3. Results and Discussion

3.1. Competitive Interaction between Fungi

The study aimed to determine the effect of competition among fungi isolated from chili. Furthermore, the most appropriate temperature for *A. oryzae* to reduce AFB$_1$ and OTA contaminations was examined. *Aspergillus oryzae* was used as it was isolated along with *A. flavus* and *A. carbonarius* from chili collected from a market in Thailand, as reported in Chuaysrinule, et al. [15].

Growth rates of single and mixed cultures are shown in Figure 1a. The single culture of *A. flavus* had the highest growth rate at 35 °C at which fungal growth rate decreased by 40.66% in mixed cultures with *A. oryzae*. Besides at 35 °C, the reduction of growth rates in mixed cultures of *A. flavus* and *A. oryzae* compared to single culture of *A. flavus* was also observed at 20 and 40 °C by 10.17% and 14.29%, respectively. At 30 °C, the growth rate of mixed cultures of *A. flavus* and *A. oryzae* was found to increase by 50.94% compared to the single culture of *A. flavus*. In *A. carbonarius*, the optimum growth rate was observed at 20 °C at which the growth rate of mixed cultures was observed to be lower by 36.70% than the single culture of *A. carbonarius* (Figure 1b). The growth inhibition by *A. oryzae* found in the current study was in agreement with the study of Alshannaq, et al. [22], which reported that cell-free culture of *A. oryzae* was able to inhibit conidial germination of *A. flavus*. They hypothesized that specific genes were involved in unknown antifungal products that could inhibit fungal growth.
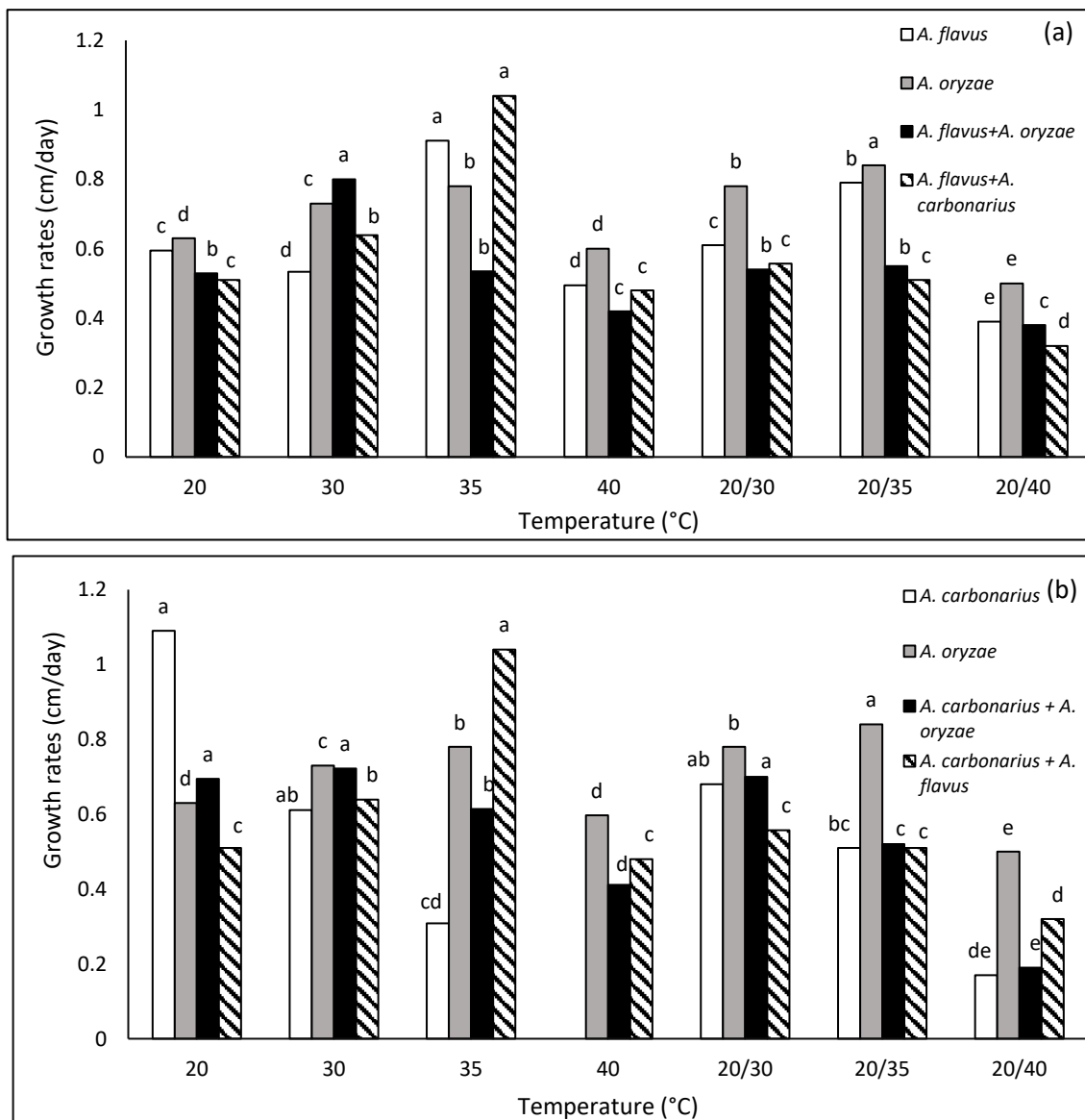
Figure 1. Growth rates, μ (cm/day), of (**a**) single culture and mixed cultures of *A. flavus,* (**b**) single culture and mixed cultures of *A. carbonarius* at static and fluctuating temperatures on CCA for 6 days. The letters (a, b, c, d, and e) indicate significant differences at *p*-value < 0.05 among 7 temperature levels on the same culture.

Moreover, the increasing growth rates of mixed cultures compared to *A. carbonarius* were found at static temperatures of 30, 35, and 40 °C by 18.03%, 96.77%, and 100%, respectively. The increasing growth rate in mixed cultures is possibly due to the temperature imposed, such as 30 °C, which was not an optimum condition for *A. flavus* and *A. carbonarius*, yet was optimum for *A. oryzae* instead, contributing to the increasing growth rates in mixed cultures. The current study observed that *A. oryzae* was optimum at the temperature of 30–35 °C. It is supported by the study of Gomi (2014), reporting that *A. oryzae* had optimum growth temperature at 32–36 °C (±1 °C), and it was unable to grow above 44 °C.

Under fluctuating temperatures, the optimum growth temperature of *A. flavus* was 20/35 °C at which mixed cultures with *A. oryzae* resulted in significantly decreasing rates by 30.38% (Figure 1a). In the single culture of *A. carbonarius,* the optimum growth rate was observed at 20/30 °C at which no significant differences in growth rates were observed between the single culture of *A. carbonarius* and mixed cultures of *A. carbonarius* and *A. oryzae*. The mixed cultures of *A. flavus* and *A. carbonarius* resulted in a lower growth rate

compared to that of the single culture of *A. flavus* and *A. carbonarius,* except at temperature of 20/40 °C. The reduction of growth rates in the mixed cultures with *A. oryzae* indicated that under fluctuating temperatures, *A. oryzae* would be more effective to inhibit the growth of *A. flavus* instead of *A. carbonarius*. It has been widely known that fungi generally were not the effective microorganisms to inhibit *A. carbonarius*. Kogkaki, et al. [45] reported that grape-associated fungal strains resulted in slight inhibition for the growth of *A. carbonarius*, particularly *A. ibericus*. However, de Almeida, et al. [46] showed that fungi had a high ability to inhibit the growth of *A. carbonarius*. The fungi used in their study were isolated from coffee beans, and they had growth inhibition potentials exceeding 70%, such as *Rhizopus oryzae, A. westerdijkiae, A. niger, A. tamari, A. fumigatus,* and *Aspergillus* sp. The possible inhibition growth mechanism is the nutrient and space competition. Control agents would utilize the nutrient before being used by the pathogen, and they have fast-growth covering a large area Besides, *A. oryzae* was possibly secreting certain substances, thereby inhibiting the growth of antagonist fungi. Skouri-Gargouri, et al. [47] reported that another nontoxigenic *Aspergillus* species, *A. clavatus,* could inhibit the growth of several plant pathogens such as *F. oxysporum* and *A. niger* through secretion of an antifungal peptide.

Under static temperatures, *A. flavus* had optimum AFB_1 production at 30 °C at which the highest inhibition of AFB_1 production by *A. oryzae* was obtained by 94% (Table 1). Meanwhile, *A. carbonarius* had an optimum temperature at 20 °C at which lower OTA production was obtained by 45% in mixed cultures with *A. oryzae*, and the highest OTA inhibition by *A. oryzae* was found at 35 °C accounted for 95%. It indicated that *A. oryzae* would be effective to inhibit AFB_1 and OTA at the static temperature of 30 and 35 °C, respectively. At 40 °C, induction of AFB_1 and OTA were observed, indicating that *A. oryzae* somehow could induce mycotoxin production under suboptimal conditions of *A. flavus* and *A. carbonarius* to produce mycotoxin. Similar trend was observed with mixed cultures of *A. flavus* and *A. carbonarius* with reduction of AFB_1 at all temperatures except at 40 °C at which AFB_1 production increased, and the concentration was higher than that in the mixed cultures of *A. flavus* and *A. oryzae*. It concluded that *A. oryzae* was significantly able to reduce AFB1 in wider range of temperature than *A. carbonarius*. Furthermore, the competition of nutrition and space with *A. carbonarius* was not sufficient to reduce AFB_1 production from *A. flavus*. Another mechanism was required to eliminate AFB_1.

Table 1. AFB_1 and OTA production (ng/g) of *A. flavus* and *A. carbonarius* in single and mixed cultures at static temperatures on CCA.

Temperature (°C)	AFB_1 (ng/g)		
	A. flavus	*A. flavus + A. oryzae*	*A. flavus + A. carbonarius*
20	2855.4 ± 9.2 [b,A]	227.2.± 20.6 [bc,B]	340.7 ± 7.4 [b,B]
30	5310.8 ± 1414.4 [a,A]	317.3 ± 85.4 [ab,B]	364.6 ± 13.7 [a,B]
35	2719.6± 707.2 [b,A]	298.2 ± 70.1 [b,B]	222.7 ± 5.1 [c,B]
40	118 ± 14.7 [c,B]	174.7 ± 25.9 [c,B]	265.1 ± 8.9 [b,A]
Temperature (°C)	OTA (ng/g)		
	A. carbonarius	*A. carbonarius+ A. oryzae*	*A. carbonarius+ A. flavus*
20	21,469.9 ± 80.2 [a,A]	11,765.1 ± 98.3 [a,C]	16,360.63 ± 67 [a,B]
30	83,38.5 ± 86.5 [bc,A]	2876.5 ± 183.7 [b,C]	3901.7 ± 105.7 [b,B]
35	7233.5 ± 970.6 [c,A]	350 ± 0.8 [c,C]	727.2 ± 170.3 [c,B]
40	NA	511.3 ± 66.7 [c,B]	998.7 ± 75.4 [c,A]

[a,b,c] Different superscript lowercase letters in the same column denote significant differences between temperature ($p < 0.05$). [A,B,C] Different superscript capital letters in the same row denote significant differences between strain ($p < 0.05$). NA, not analyzed since no growth was observed.

Under fluctuating temperatures, *A. oryzae* reduced AFB_1 at 20/30 °C by 100% and OTA at 20/30, 20/35, and 20/40 °C by 34, 56, and 23%, respectively (Table 2). However,

AFB$_1$ increased in the mixed cultures with *A. oryzae* at 20/35 and 20/40 °C. It indicated that fluctuating temperatures caused *A. oryzae* to inhibit or induce AFB$_1$ and OTA. The shifts at suboptimal temperatures would cause *A. flavus* to dominate the competition of nutrition or space over *A. oryzae,* leading to higher AFB$_1$ production in *A. flavus* compared to the ability of *A. oryzae* to reduce it. Meanwhile, fluctuating temperatures inside and outside the optimum range still resulted in OTA inhibition by *A. oryzae* despite a lower inhibition rate compared to static temperatures. Inhibition rate at static temperatures ranged 45–95%, while that at shifting temperatures ranged from 23–56%. Following mixed cultures with *A. carbonarius* or *A. flavus*, production of AFB$_1$ and OTA, respectively, decreased at 20/30 °C and increased at 20/35 and 20/40 °C.

Table 2. AFB$_1$ and OTA production (ng/g) of *A. flavus* and *A. carbonarius* in single and mixed cultures at fluctuating temperatures on CCA.

Temperature (°C)	AFB$_1$ (ng/g)		
	A. flavus	*A. flavus* + *A. oryzae*	*A. flavus* + *A. carbonarius*
20/30	1495.9 ± 94.4 [ab,A]	ND	194.7 ± 1.53 [b,B]
20/35	295 ± 30.6 [b,A]	416.3 ± 22.5 [a,A]	324 ± 12.18 [ab,A]
20/40	251 ± 17 [b,A]	329.6 ± 25.1 [ab,A]	350.3 ± 21.5 [a,A]
Temperature (°C)	OTA (ng/g)		
	A. carbonarius	*A. carbonarius* + *A. oryzae*	*A. carbonarius* + *A. flavus*
20/30	12,799.3 ± 3000.9 [a,A]	8394 ± 354.7 [a,C]	11,957.3 ± 300.6 [a,B]
20/35	7725.5 ± 1233.7 [b,A]	3409.4 ± 180.2 [b,C]	8042.3 ± 572.1 [b,B]
20/40	2080.5 ± 239.6 [c,B]	1604.2 ± 122.9 [c,C]	3693.4 ± 74.4 [c,A]

[a,b,c] Different superscript lowercase letters in the same column denote significant differences between temperatures ($p < 0.05$). [A,B,C] Different superscript capital letters in the same row denote significant differences between strains ($p < 0.05$). ND, not detected.

In the current study, *A. oryzae* was able to reduce AFB$_1$ and OTA contamination. This result is in agreement with the study by Lee et al. [23], who reported that *A. oryzae* isolated from Meju, a traditional Korean fermented soybean starter, has the potential to control AFB$_1$ in foods and feeds. Furthermore, Alshannaq, et al. [22] showed similar results, in which *A. oryzae* strains and *A. oryzae* cell-free PDB fermentate could control *A. flavus* growth and aflatoxin contamination in peanuts. The ability of *A. oryzae* to reduce AFB$_1$ was possibly due to several reasons, including (1) superior abilities of *A. oryzae* to sequester nutrients, (2) production of metabolites that might inhibit AFB$_1$ production, (3) non-protein substances that naturally occurred in fermentate, and (4) specific genes involved in unknown antifungal products inhibiting growth and mycotoxin production in *A. flavus*. In OTA, the reduction by *A. oryzae* could be caused by several factors, including (1) growth limitation of *A. carbonarius* by co-culture with *A. oryzae* leading to reduction of OTA production, (2) consumption by *A. oryzae*, the nutrients required to synthesize OTA, and (3) OTA degradation by *A. oryzae*. Several fungi could degrade OTA due to carboxypeptidase A activity such as *A. niger, R. oryzae, Alternaria, Cladosporium,* and *Trichoderma* [48]. At 40 °C, 20/35 °C, and 20/40 °C, the production of AFB$_1$ was higher in mixed cultures than in the single culture of *A. flavus*. In OTA, the rise in production was observed at 40 °C. Despite the non-significant increase in concentrations, it was still worth noting to investigate. The increase of mycotoxin production was presumably due to competition with *A. oryzae* and temperature stress. *A. oryzae* caused *A. flavus* and *A. carbonarius* to produce AFB$_1$ and OTA as their defense mechanisms against the threat. Fox, et al. [49], Magan, et al. [50] suggested that mycotoxins be able to act as chemical signals in communication, a competitive weapon to defend the habitat, or to inhibit the growth of competitors in the same niche. Furthermore, the presence of competitors is essential for mycotoxin production. It is supported by Horn, et al. [51], who reported that serial transfers on culture media

within laboratory conditions, in which they lacked interaction among fungi and natural stressed conditions, resulted in the loss of aflatoxins production in *A. flavus*. Temperature stress also contributed to the increased production of AFB_1 and OTA because it can activate the mycotoxin biosynthesis genes [52,53].

Overall, *A. oryzae* seems to be appropriate fungi to inhibit mycotoxigenic fungi and eliminate AFB_1 and OTA. Moreover, it was much more effective to inhibit the growth of *A. flavus* instead of *A. carbonarius* both at static and fluctuating temperatures on CCA. In terms of mycotoxin inhibition at static temperatures, the effectiveness of *A. oryzae* in inhibiting AFB_1 and OTA production was based on temperature. The temperatures of 30 and 35 °C were optimum conditions for AFB_1 and OTA inhibition by *A. oryzae*, respectively. Under fluctuating temperatures, *A. oryzae* was much more effective in inhibiting OTA instead of AFB_1 as reduction of OTA was observed under all fluctuating temperature programs. In AFB_1, the production was only reduced at 20/30 °C.

3.2. Interaction between Toxigenic and Non-Toxigenic Species on Chili-Based Agar

Since all the fungi used were isolated from chili, this part was conducted to determine the effectiveness of *A. oryzae* in inhibiting AFB_1 and OTA on chili-based agar. In the current part of the study, dual cultures were carried out by inoculating spore suspension of two fungi in two different areas on the same agar medium; thus, toxigenic fungi (*A. flavus* or *A. carbonarius*) and non-toxigenic fungi (*A. oryzae*) each have growth diameter. The fluctuating temperatures were performed by manually changing temperatures from 20 °C to 40 °C. Each temperature was imposed for 15 h and 9 h, respectively, for 4 days after pre-incubation at 20 °C for 3 days. The media was used with 2 a_w levels, 0.92 and 0.97 which were selected because water activity of more than 0.90 resulted in high production of AFB_1 and OTA [15,54,55].

The growth of *A. flavus* at the end of incubation time was observed to be higher at 20 °C than at 40 °C (Figure 2a). By the presence of *A. oryzae*, the growth of *A. flavus* was not significantly different at 20 °C and 40 °C (Figure 2b). It indicated that low water activity (0.92 a_w) resulted in the inability of *A. oryzae* to inhibit the growth of *A. flavus* at both static temperatures of 20 °C and 40 °C. Meanwhile, growth of *A. flavus* at 20 and 40 °C was observed lower after dual cultures with *A. oryzae* at 0.97 a_w (Figure 3). It showed that under static temperature, particularly temperatures of 20 and 40 °C, *A. oryzae* can inhibit the growth of *A. flavus* at water activity more than 0.92 a_w, specifically 0.97 a_w. On the contrary, previous study reported that *A. oryzae* can grow on media with water activity above 0.8 [56]. According to that, *A. oryzae* is supposed to grow and inhibit the growth of *A. flavus* at 0.92 a_w. This condition is presumably due to dual cultures with *A. flavus*, which hinder the growth phase of *A. oryzae*.
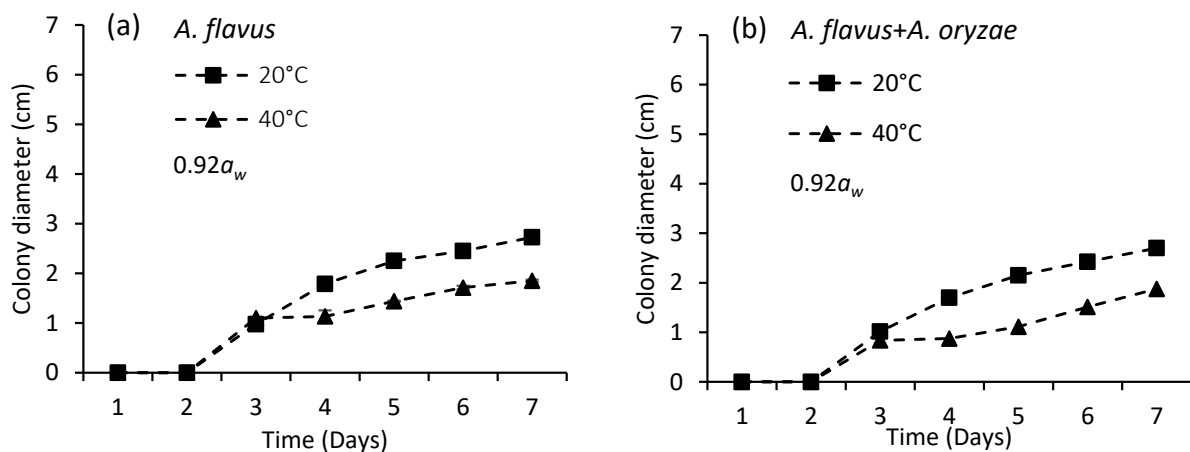

Figure 2. Growth of *A. flavus* in (**a**) single and (**b**) dual cultures with *A. oryzae* incubated under static temperatures of 20 °C and 40 °C for 7 days at 0.92 a_w on chili-based agar.
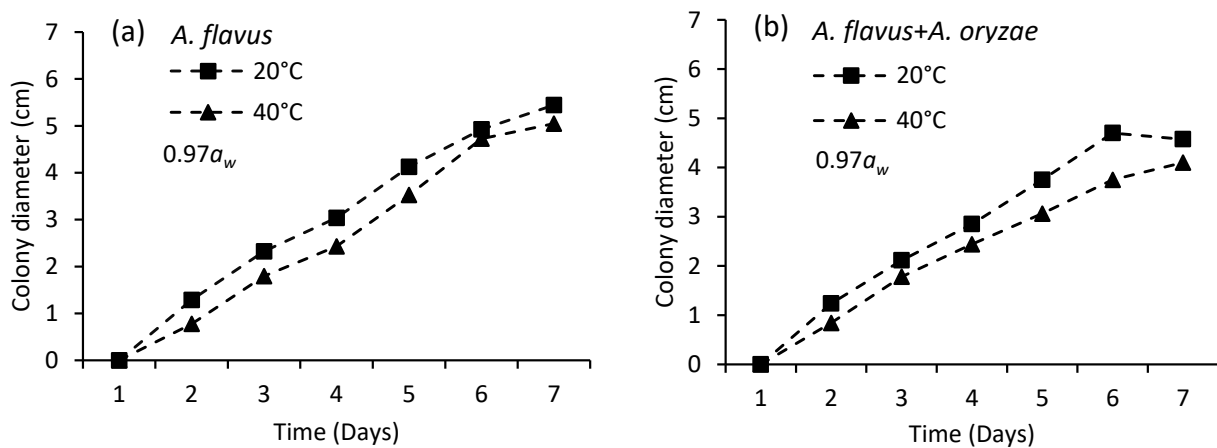
Figure 3. Growth of *A. flavus* in (**a**) single and (**b**) dual cultures with *A. oryzae* incubated under static temperatures of 20 °C and 40 °C for 7 days at 0.97 a_w on chili-based agar.

In *A. carbonarius,* no growth was observed at static temperatures of 40 °C, at 0.97 a_w and 0.92 a_w. At 20 °C and 0.92 a_w, there were no significant differences observed between the growth of *A. carbonarius* in single and dual cultures with *A. oryzae* (Figure 4a). By increasing the water activity to 0.97 a_w, *A. carbonarius* in single and dual cultures was also increasing at the same temperature. Under such condition, *A. carbonarius* in single culture was observed to be higher than in dual culture (Figure 4b). It indicated that *A. oryzae* would be adequate to inhibit the growth of *A. carbonarius* at a high water activity (0.97 a_w).
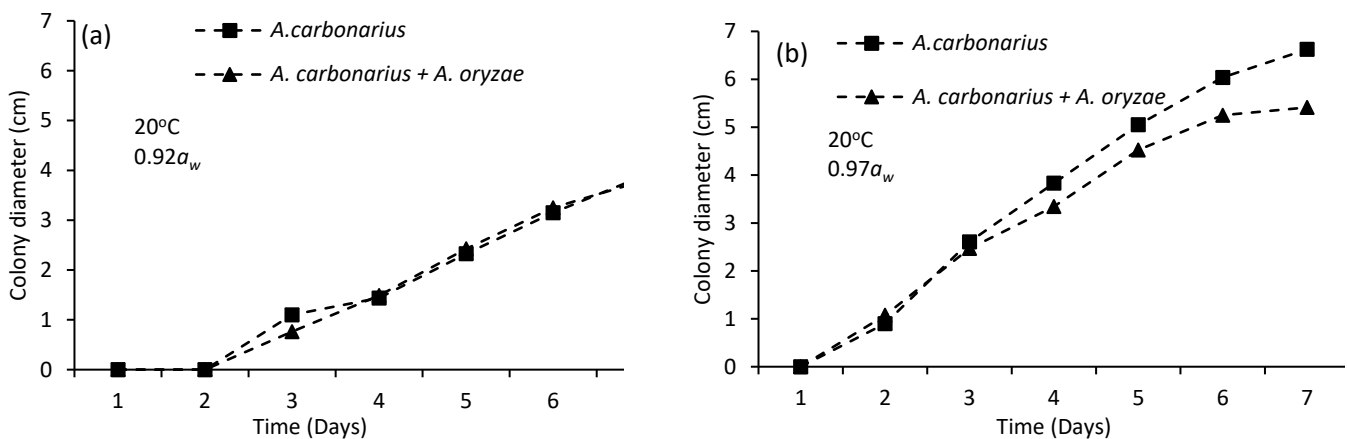

Figure 4. Growth of *A. flavus* in single and dual cultures with *A. oryzae* incubated under fluctuating temperatures of 20/40 °C with 15 h/9 h cycling time for 7 days, at (**a**) 0.92 a_w and (**b**) 0.97 a_w on chili-based agar.

No toxin was produced under static temperatures on the fourth day of incubation as no AFB$_1$ and OTA were detected. After incubation for 7 days, AFB$_1$ was detected at 20 °C and 40 °C (Table 3). At 20 °C, AFB$_1$ was detected only in the single culture of *A. flavus* at water activity of 0.97 a_w, while no AFB$_1$ was observed in dual cultures. It resulted in inhibition of AFB$_1$ by *A. oryzae* (100%). At 40 °C, AFB$_1$ was detected at 0.92 a_w and 0.97 a_w, at which AFB$_1$ concentrations were observed to be lower by 9.37% and 14.43%, respectively, in dual cultures compared to those in the single culture. Meanwhile, OTA was only detected at a static temperature of 20 °C, at 0.92 and 0.97 a_w. Under those conditions, the concentrations of OTA were observed to be lower in dual cultures with *A. oryzae* in comparison to single culture of *A. carbonarius*. At 20 °C with the water activity of 0.92 and 0.97 a_w, the reduction of OTA in dual cultures was observed by 43.33% and 68.42%, respectively. Lower concentrations of AFB$_1$ and OTA after dual cultures with *A. oryzae*

indicated that *A. oryzae* could reduce AFB_1 and OTA, either by inhibition of mycotoxin production or degradation of mycotoxin. *A. oryzae* would be optimal to inhibit AFB_1 and OTA at low temperature and high water activity (20 °C, 0.97 a_w).

Table 3. AFB_1 and OTA production by *A. flavus* and *A. carbonarius* in single and dual cultures with *A. oryzae* on chili-based agar with 0.92 a_w and 0.97 a_w for 4 and 7 days at 20 °C and 40 °C.

Temperature (°C)	a_w	Strains	AFB_1 (ng/g)		Conversion Rate (%) *
			Day 4	Day 7	
20	0.92	*A. flavus*	ND	ND	
		Dual cultures	ND	ND	-
	0.97	*A. flavus*	ND	912.57 ± 4.19	
		Dual cultures	ND	ND	−100
40	0.92	*A. flavus*	ND	3503.23 ± 97.94 [a]	
		Dual cultures	ND	3174.87 ± 14.04 [b]	−9.37
	0.97	*A. flavus*	ND	2641.76 ± 170.3 [a]	
		Dual cultures	ND	2286.23 ± 300.6 [a]	−14.43

Temperature (°C)	a_w	Strains	OTA (ng/g)		Conversion rate (%) *
			Day 4	Day 7	
20	0.92	*A. carbonarius*	ND	141,354.6 ± 89.06 [a]	
		Dual cultures	ND	80,253.01 ± 52.93 [b]	−43.23
	0.97	*A. carbonarius*	ND	446,221.61 ± 30.26 [a]	
		Dual cultures	ND	140,929.05 ± 62.78 [b]	−68.42
40	0.92	*A. carbonarius*	NA	NA	
		Dual cultures	NA	NA	-
	0.97	*A. carbonarius*	NA	NA	
		Dual cultures	NA	NA	-

[a,b] Different superscript lowercase letters denote significant differences between strains within the same temperature and a_w ($p < 0.05$). * Plus (+) or minus (−) indicates either induction or inhibition of mycotoxin, respectively, after mixed cultures with *A. oryzae*. NA, not analyzed since no growth was observed. ND, not detected.

Under fluctuating temperatures of 20/40 °C with 0.92 a_w, there were no significant differences between the growth of *A. flavus* in single and dual cultures during incubation (Figure 5a). It was also observed at water activity of 0.97 (Figure 5b), indicating that fluctuating temperatures would inhibit *A. oryzae* from controlling the growth of *A. flavus* even after the water activity had been raised to 0.97 a_w. However, higher growth of *A. flavus* in dual cultures was observed at 0.97 a_w than at 0.92 a_w at the end of incubation-day, which indicated that high water activity was favorable for the growth of *A. flavus* under fluctuating temperatures.

In *A. carbonarius*, no growth was observed in fluctuating temperatures of 20/40 °C and 0.92 a_w. At 20/40 °C and 0.97 a_w, the growth of *A. carbonarius* in dual cultures was observed to be higher than that in single culture (Figure 6). At the end of incubation, the growth of *A. carbonarius* in single and dual cultures under fluctuating temperatures was observed to be lower than their growth under static temperature (20 °C and 0.97 a_w, Figure 5b). It indicated that fluctuating temperatures could cause stress conditions in *A. carbonarius*. Hence, the growth was resisted. However, the presence of *A. oryzae* could help *A. carbonarius* grow despite the stress-inducing situation, considering higher growth of *A. carbonarius* that was observed in dual cultures than in single culture under fluctuating temperatures. Furthermore, colonies of *A. carbonarius* started to be visible after the second day of incubation at static temperature, while their colonies were visible on the fourth day of incubation under fluctuating temperatures. It indicated that fluctuating temperatures caused *A. carbonarius* in both single and dual cultures to grow slower compared to the growth under static temperature of 20 °C.
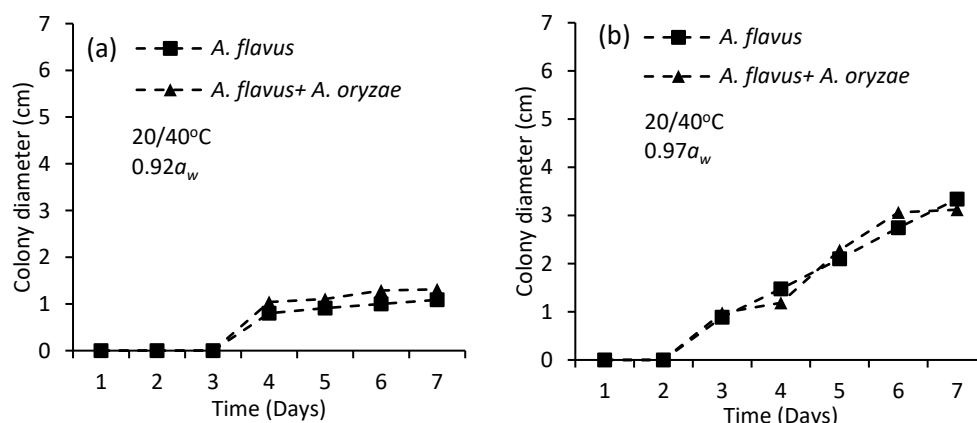
Figure 5. Growth of *A. flavus* inoculated in single and dual cultures with *A. oryzae* incubated under fluctuating temperatures of 20/40 °C with 15 h/9 h cycling time at (**a**) 0.92 a_w and (**b**) 0.97 a_w for 7 days on chili-based agar.
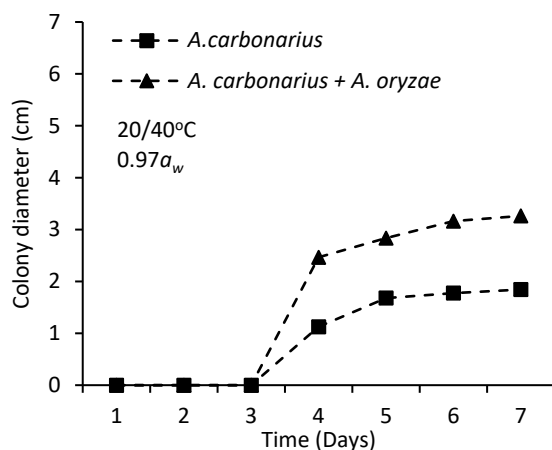

Figure 6. Growth of *A. carbonarius* inoculated in single and dual cultures with *A. oryzae* incubated under fluctuating temperatures of 20/40 °C with 15 h/9 h cycling time at 0.97 a_w for 7 days on chili-based agar.

Under fluctuating temperatures, production of AFB$_1$ at 0.92 a_w was induced with the presence of *A. oryzae* (Table 4). However, the production at 0.97 a_w was inhibited instead. It indicated that under fluctuating temperatures, the ability of *A. oryzae* to inhibit mycotoxin would be affected by water activity. With the current fluctuating temperature scenario, *A. oryzae* could not inhibit AFB$_1$ at water activity of 0.92 a_w or less than 0.97 a_w. It is in agreement with the study by Peromingo, et al. [30], which demonstrated that *Debaryomyces hansenii* could stimulate aflatoxins production by *A. parasiticus* at 0.99 a_w, whereas a significant reduction of aflatoxins was observed at 0.92 a_w. In a study by La Penna, et al. [57], *Kluyveromyces* spp. were able to completely inhibit aflatoxin production of *A. flavus* and *A. parasiticus* at high a_w (0.994 and 0.982), but they could increase aflatoxin levels with certain *Aspergillus* at low a_w (0.955 and 0.937). In OTA, fluctuating temperatures caused *A. oryzae* to inhibit the production at 0.97 a_w by 42.23%.

Table 4. AFB$_1$ and OTA production by *A. flavus* and *A. carbonarius* on single and dual cultures with *A. oryzae* on chili-based agar with 0.92 a$_w$ and 0.97 a$_w$ for 4 and 7 days at 20/40 °C.

Temperature (°C)	a$_w$	Strains	AFB$_1$ (ng/g) Day 4	AFB$_1$ (ng/g) Day 7	Conversion Rate (%) *
20/40	0.92	*A. flavus*	ND	4093.15 ± 190 [b]	
		Dual cultures	ND	4512.01 ± 21.03 [a]	+10.23
	0.97	*A. flavus*	3804.52 ± 126.23	4837.44 ± 68.02 [a]	
		Dual cultures	ND	37.49 ± 16.90 [b]	−99.23

Temperature (°C)	a$_w$	Strains	OTA (ng/g) Day 4	OTA (ng/g) Day 7	Conversion rate (%) *
20/40	0.92	*A. carbonarius*	NA	NA	
		Dual cultures	NA	NA	-
	0.97	*A. carbonarius*	ND	37,748.06 ± 37.65 [a]	
		Dual cultures	ND	21,806.01 ± 16.49 [b]	−42.23

[a,b] Different superscript lowercase letters denote significant differences between strains within the same temperature and a$_w$ ($p < 0.05$). NA, not analyzed since no growth was observed. ND, not detected. * Plus (+) or minus (−) indicates either induction or inhibition of mycotoxin, respectively, after dual cultures with *A. oryzae* on 7-day incubation.

4. Conclusions

A. oryzae was optimum to reduce AFB$_1$ and OTA contamination in crops at high water activity and low temperature (0.97 a$_w$, 20 °C). In some cases in which the fluctuating temperatures could not be avoided during storage or shipment of crops, *A. oryzae* was still able to inhibit AFB$_1$ and OTA production despite decreasing inhibition rate in comparison to static temperatures. However, the current study suggested that it was used to inhibit OTA instead of AFB$_1$. Further studies could address a greater variety of fungi and mycotoxin that *A. oryzae* could degrade under various abiotic stress that possibly occurred during postharvest to have a comprehensive study for the degradation of mycotoxins.

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