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Review

Recent progress of the effect of environmental factors on *Aspergillus flavus* growth and aflatoxins production on foods

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Abstract

The contamination of *Aspergillus flavus* and subsequent aflatoxins (AFs) has been considered as one of the most serious food safety problems due to their acute and chronic adverse effects on humans and animals. This review collects the available information from recent years on the effect of the major environmental factors such as water activity (a_w), temperature, CO_2, and pH on the fungal growth, the expression of AFs-related genes, and AFs production by *A. flavus* on foods. In particular, the relationship between the relative expression of key regulatory (*aflR* and *aflS*) and structural genes (*aflD*, *aflO*, *aflQ*, etc.) and AFs production under different environmental conditions are collected and discussed. The information collected in this review can be used to design control strategies of *A. flavus* and AFs contamination in practical applications, primarily during storage and processing. These data suggest that integrating various post-harvest methods with synergistic functions may be more efficient for the control of *A. flavus* growth and AFs production, although the individual environmental factors alone have an impact.

Key words: *Aspergillus flavus*; aflatoxin; water activity; temperature; CO_2.

Introduction

The moldy contamination of staple foods such as cereals has been regarded as one of the most serious food safety problems due to their acute and chronic negative effects on humans and animals (Medina *et al*., 2014). Certain molds such as *Aspergillus flavus* and *Aspergillus parasiticus* grown in corn, peanuts, and nuts produce aflatoxins (AFs). AFs have been classified as carcinogens in Group 1 by the International Agency for Research on Cancer (IARC, 1993, 2002). They are estimated to induce up to 28% of the total worldwide cases of hepatocellular carcinoma (HCC), the most common form of liver cancer (Liu *et al*., 2012; Wu, 2014). Moreover, AFs also inevitably cause acute intoxication, immune suppression, and growth retardation in children (Groopman *et al*., 2008). Among AFs, aflatoxin B_1 (AFB_1) was the most toxic one. The main source of exposure to AFs is the ingestion of contaminated food and feed. Therefore, the high threat of AFs to the health of humans and animals has resulted in strict legislative limits in most countries and regions of the world for AFs and AFB_1 in a wide range of foodstuffs and feeds (Commission of the European Communities, 2010; US Food and Drug Administration, 2010).

Aspergillus flavus is the predominant fungal species contaminating foodstuffs and feeds and producing AFs worldwide. It is also the main contaminant during the food storage since its ability to produce AFs and its potential to persist as a pathogen and saprophyte

in the food supply before and after harvest (Lahouar *et al.*, 2015). *Aspergillus flavus* is a xerophilic fungus that has developed physiological mechanisms that adapt to environmental stress factors like low water activity (a_w), allowing them to compete and often dominate other fungal communities (Nierman, 2008; Medina *et al.*, 2015). The competitive advantage is because its metabolic plasticity allows it to produce a range of extracellular hydrolases, secondary metabolites, and volatiles (Magan and Aldred, 2007). Therefore, preventing and controlling the fungal growth and AFs production by *A. flavus* are essential and urgent to ensure the food safety and security in the world.

To eliminate AFs contamination in food chain, the most useful and cost-effective strategy is to create an adverse environment for *A. flavus* growth and AFs production. The growth of *A. flavus* at the phenotypic level and the AFs production were observed to be associated with several environmental factors such as a_w, temperature, storage time, composition of the substrate, carbon and nitrogen source, pH, light, content of oxygen (O_2) and carbon dioxide (CO_2), loss of grains' integrity caused by insects or mechanical/thermal damage, and the interaction between fungal species that share the same ecological environment (Dantigny *et al.*, 2005; Vaamonde *et al.*, 2006; Astoreca *et al.*, 2014; Medina *et al.*, 2015). Regarding abiotic factors, a_w and temperature and their interactions have been demonstrated to be the major aetiological determinants in regulating fungal growth and secondary metabolites production (Mannaa and Kim, 2017; Medina *et al.*, 2017). As an aerobic fungus, *A. flavus* growth and subsequent AFs production are highly influenced by CO_2 with certain levels (Peleg *et al.*, 1988; Mousa *et al.*, 2016). AFs are biosynthesized by 29 genes located in a 75 Kb gene cluster in *A. flavus*, including the two regulatory genes (*aflS* and *aflR*) and the structural genes such as *aflD*, *aflM*, and *aflO* (Liu *et al.*, 2017). The biosynthesis of AFs is also regulated by genes encoding the velvet proteins such as *veA* and *laeA*, as well as developmental genes modulating morphology, conidiation, or sclerotia formation like *brlA* and *abaA* (Caceres *et al.*, 2016). Some oxidative stress-related genes and cellular signal mediator genes such as r*asA*, *msnA*, *mtfA*, and oxylipin's biosynthetic genes (Tsitsigiannis and Keller, 2007); G-protein receptor genes (Affeldt *et al.*, 2014); and osmotic-adaptation response gene *sakA* (Tumukunde *et al.*, 2019) can also influence AFs biosynthesis (Caceres *et al.*, 2016).

The expression of AF pathway genes is also influenced by some environmental factors. In particular, the a_w × temperature interactions are related to the ratio of the two key regulatory genes (*aflS/aflR*). And the higher ratio of *aflS/aflR* would relate to the higher level of AFs production (Schmidt-Heydt et al., 2009, 2010; Abdel-Hadi et al., 2010, 2012; Medina *et al.*, 2014). Tumukunde *et al.* (2019) indicated that low a_w was negatively related to the production of AFB$_1$ on corn and peanut kernels. Low a_w reduced the expression of *AfsakA*, which affected the conidiation and AFs biosynthesis. Moreover, the expression of 11 development-related genes increased under 0.99 a_w treatment (Zhang *et al.*, 2014). As for high temperature, *aflS* and *aflR* were inhibited. The expression level of *aflS/aflR* seems to control the transcriptional activation of the AFs cluster (Yu *et al.*, 2011). Compared with 37°C, the transcript abundance of 30 AFs biosynthesis genes was much higher at 30°C, and most genes were up-regulated at both protein and transcription levels at 28°C (Bai *et al.*, 2015). Taken together, the environmental factors have significant effects on *A. flavus* growth, the expression of AF biosynthetic genes, and AFs production.

Therefore, controlling the environmental factors is the most useful, cost-effective, practical, and green environmental strategy to prevent and eliminate *A. flavus* and AF contaminations. This paper is aimed at reviewing the effects of the major environmental factors on fungal growth and AFs production by *A. flavus*. Several studies have revealed the effect of environmental factors on fungal growth, genes expression, and AFs production. In order to avoid duplication with that presented by Medina *et al.* (2014), studies that displayed the effects of environmental factors in the above-mentioned review are not thoroughly discussed. The following subsections describe the studies published after 2014 and papers not presented in the aforementioned review. This review may provide the basic data for optimizing the environmental conditions to control AFs contamination on foodstuffs and feeds, especially during the storage.

Effect of a_w and temperature on *A. flavus* growth, gene expression, and AFs production on grains

Plenty of literatures have reported that the two key environmental parameters, a_w and temperature, play important roles in modulating the *A. flavus* growth and AFs production on foods (O'Brian *et al.*, 2007; Schmidt-Heydt et al., 2009, 2010; Yu *et al.*, 2011; Marroquín-Cardona *et al.*, 2014; Bai *et al.*, 2015). Among foods, grains are the most susceptible to the infection of *A. flavus* and the subsequent AFs contamination (Guo *et al.*, 1998). Regarding AFs production, various grains have a different sensitivity to a_w and temperature. Moreover, on the same grain, the optimal conditions for AFs production were significantly influenced by the *A. flavus* strains, incubation time and status of grains, etc.

Mannaa and Kim (2018) investigated the effects of different relative humidities (RHs; 12, 44, 76, and 98%) and temperatures (10, 20, 30, and 40°C) on major grain fungal populations including *A. flavus* and some other fungi. They indicated that the populations of all tested fungi in inoculated rice grains were significantly enhanced by both increased RH and temperature. In addition, multiple linear regression analysis revealed that one unit increase of temperature resulted in greater effects than that of RH on fungal populations.

Mousa *et al.* (2013) conducted a study to model the radial growth rate and to assess AFs production by *A. flavus* as a function of a_w 0.82–0.92 and temperature 12 –42°C on polished and brown rice. They found that the brown rice is more susceptible to fungal invasion and AFs production than polished rice. The optimum conditions for *A. flavus* growth and AFs production in brown rice were both at 0.92 a_w and 30°C. Fungal growth and AFs were undetectable at 0.82 a_w on polished rice while they were both detected at this a_w under 25–35°C on brown rice. At the temperature range of 25–30°C, the minimum a_w values for AFs production on polished rice and brown rice were 0.84 and 0.82, respectively. The highest levels of AFs were observed at the highest a_w 0.90–0.92 at 20°C after 21 days of incubation on both types of rice. However, at 7 and 14 days of incubation, the highest level of AFs was observed at a_w 0.92 and 30°C on polished rice. On brown rice, the optimal condition for AFs production at 7 and 14 days of incubation was a_w 0.92 × 25°C and a_w 0.92 × 30°C, respectively. When temperature reached to 40°C, only a small amount of AFs were detected during the 3 weeks of incubation, and both types of rice were only detectable at 0.92 a_w.

Similarly, Choi *et al.* (2015) showed that brown Korean rice was sensitive to *A. flavus* growth and AF production compared with rough Korean rice and white Korean rice. Regardless of the degree of milling of Korean rice, the optimum growth rate during 120 day storage was at 85% RH/30°C and 97% RH/21°C. The highest population of *A. flavus* and highest amount of AFB$_1$ were observed at 97% RH/21°C and on inoculated brown rice. Trace amounts of AFB$_1$ were detected during 10 day storage at 85% RH/21°C and in all three non-inoculated types of rice.

Lv *et al.* (2019) investigated the effect of a_w (0.92–0.96) and temperature (28–37°C) on the fungal growth and AFB_1 production by *A. flavus* on polished rice and paddy. The results indicated that AFB_1 production on polished rice can occur over a wider range of temperature × a_w levels than that on paddies. *Aspergillus flavus* grew better at 0.92–0.96 a_w and 28–37°C on polished rice, and the highest level of AFB_1 was observed at 33°C and 0.96 a_w after 7 days of incubation. In comparison to the optimal temperature of 33°C, all tested genes of *A. flavus* on polished rice were significantly up-regulated at 25°C under 0.96 a_w. Compared with a_w 0.96, most of structural genes of pathway were significantly down-regulated at a_w 0.90 and 0.99 under 33°C, although two regulatory genes (*aflR* and *aflS*) were up-regulated at a_w 0.90. For the *A. flavus* growth on paddy, the optimal temperature and a_w were 37°C and 0.94 within the tested range, respectively. Moreover, the highest concentration of AFB_1 on paddy was observed at 0.92 a_w and 37°C after 7 days of incubation.

Lahouar *et al.* (2016) evaluated the impact of a_w (between 0.85 and 0.99), temperature (15, 25, and 37°C), and incubation time (7, 14, 21, and 28 days) on *A. flavus* growth and AFB_1 production by three *A. flavus* isolates (8, 10, and 14) inoculated on sorghum grains. Results showed that the optimal conditions for growth and AFB_1 production were at 0.99 a_w and 37°C. For mycelial growth, the needed minimum a_w was 0.91 at 25 and 37°C. AFB_1 accumulation could be avoided by storing sorghum at low a_w levels (≤0.91 a_w) or at low temperature (15°C).

Astoreca *et al.* (2014) studied the interaction of a_w (0.80–0.98), temperature (10–40°C), and incubation time (7, 14, 21, and 28 days) on the co-production of AFB_1 and cyclopiazonic acid (CPA) by *A. flavus* isolated from corn on Czapek yeast agar (CYA) and corn extract agar (CEM). They found the AFB_1 production occurred more favourably on CYA while the maximum levels of CPA were observed on CEM. The minimum level of a_w for both toxins production was 0.83 with the tested a_w. The maximum amount of AFB_1 was observed at 0.96 a_w and 30°C after 21 days of incubation, regardless of the isolates and media. Although they belong to three different chemotypes: chemotype I (AFB_1+/CPA+), chemotype III (AFB_1+/CPA–), and chemotype IV (AFB_1–/CPA+), respectively, the three isolates do not differ in the response to the environmental factors (a_w and temperature). Moreover, the limiting and optimum conditions for AFB_1 and CPA production were similar on both media.

Bernáldez *et al.* (2017) investigated the impact that interactions between a_w and temperature may have on growth, the expression of *aflR*, and AFB_1 production by *A. flavus* on a maize-based medium. They found that there were some differences between lag phases and growth rates of *A. flavus*. The optimum condition for *A. flavus* growth on maize was 0.99 a_w and 30°C and the maximum AFB_1 production on maize was observed at 0.98 a_w and 30°C. *Aspergillus flavus* growth was completely inhibited at 0.90 a_w and 20°C. Both a_w and temperature significantly influenced the relative expression of *aflR* gene and AFB_1 production. However, the influences on AFB_1 production were not consistent with the effects on gene expression and growth. These results suggested that the *aflR* expression was not a good indicator of AFB_1 production alone. Therefore, further molecular studies of other AFs biosynthetic genes should be conducted.

Based on the research by Krulj *et al.*, (2019), the optimal conditions for AFB_1 biosynthesis were observed at 30°C in the temperature ranges of 15–37°C and the a_w levels at (0.85–0.99) in the inoculated shell-less and shelled cereals. Moreover, the shell has a higher amount of AFB_1 in hull-less grain than the dehulled grains

and the AFB_1 content in the hull was even 10–170 times higher than the grain, indicating the shell has a protective effect.

Effect of a_w and temperature on *A. flavus* growth, gene expression, and AFs production on peanuts

Peanut (*Arachis hypogaea* L.) is a globally important economic and oilseed crop worldwide. However, peanuts contamination with AFs and aflatoxigenic *A. flavus* is regarded as the most serious problem in the world (Williams *et al.*, 2004; Liu *et al.*, 2017). Liu *et al.* (2017) investigated the effect of a_w (0.85–0.99) and temperature (15–42°C) on fungal growth, the expression of AF biosynthetic genes on un-autoclaved peanut kernels. The results indicated that AFB_1 production in peanut kernels can occur over a wider range of a_w and temperature compared with formula media and peanut media. *Aspergillus flavus* showed a lower growth rate at ≤0.85 a_w or ≤20°C. The optimal conditions for *A. flavus* growth on peanuts kernels were 0.98 a_w and 37°C. The highest amount of AFB_1 was observed at 0.96 a_w and 28°C.

Moreover, the expression of AF-related and growth-related genes was significantly modulated by a_w and temperature. At 0.92 a_w, 16 of the 25 genes had the highest expression levels at 28°C, whereas 9 genes had the highest expression levels at 37°C. In addition, all AF biosynthetic pathway genes were down-regulated at 42°C compared with 37°C. Compared with 0.99 a_w, all the pathway genes and *laeA* were up-expressed at a_w of 0.96 under 28°C. In particular, the ratio of *aflS*/*aflR* was positively correlated with the AFB_1 production. The expressions of *laeA* and *brlA* were positively associated with AFB_1 production and fungal growth, respectively.

Effect of a_w and temperature on *A. flavus* growth and AFs production on tree nuts

Tree nuts are commodities with moderate to high risk of AFs contamination because they are produced at environmental conditions favouring fungal growth and AFs production by aflatoxigenic fungi especially *A. flavus* (Arrus *et al.*, 2005; Gallo *et al.*, 2016). The incidence of AFs contamination in nuts is low; however, their levels vary widely and can produce high levels in a small number of nuts (Campbell *et al.*, 2003).

For the pistachio nuts, the highest amount of radial growth rate of *A. flavus* and AFB_1 production was obtained at 0.93 a_w and 30°C using a full factorial design with different moisture content levels (10, 15, 20, 25, and 30%) and incubation temperatures (10, 15, 20, 25, 30, 37, and 42°C) (Marín *et al.*, 2012). They also found that the limited AFs levels in pistachio nuts by European Commission would be surpassed in a period as short as 1 month if pistachio nuts reach 20°C, unless moisture content is ≤10%. However, this conclusion is not accurate because it was drawn from a single *A. flavus* strain. As we all know, the level of mycotoxins produced is highly variable even if only considering mycotoxin-producing strains in a species (Hua *et al.*, 2012).

Gallo *et al.* (2016) evaluated the effects of different combinations of a_w (0.90, 0.93, 0.96, and 0.99 a_w) and temperature (20, 28, and 37°C) on *A. flavus* growth, AFB_1 production, and expression of the two regulatory genes and two structural genes on an almond medium solidified with agar. The maximum amount of fungal biomass and AFB_1 production was observed at 0.96 a_w and 28°C. At the driest tested conditions (0.90 and 0.93 a_w), no fungal growth and AFB_1 production were observed at 20°C. At 20 and 37°C, the yield of AFB_1 was reduced by 70%–90% or completely suppressed, depending on a_w. Both regulatory genes (*aflR* and *aflS*) showed high

expression at maximum (28°C) and minimal (20 and 37°C) AFB_1 production. In contrast, the two structural genes (*aflD* and *aflO*) showed high expression only at the maximum AFB_1 production (28°C and 0.96–0.99 a_w). Based on this, temperature appears to be a key factor affecting AFs production, which was strictly correlated with the expression of the structural genes (*aflD* and *aflO*), but not to that of the two regulatory genes. This result suggests that some post-transcriptional regulatory processes are involved in modulating AFs biosynthesis.

Prencipe *et al.* (2018) evaluated the drying temperatures (from 30 to 50°C) on *A. flavus* growth and AFs production in chestnuts and indicated that the optimal temperature for fungal growth was 30°C, whereas the highest concentrations of AFB_1 and AFB_2 were obtained at 40°C. At this temperature, *A. flavus* was under suboptimal conditions for growth (0.78 a_w), but AFs biosynthesis was under the optimal conditions. When the drying temperatures reached 45–50°C, AFs production was completely inhibited. Drying at 45°C for 7 days (0.64 a_w) could be a promising strategy to effectively control both *A. flavus* growth and AFs production.

Effect of a_w and temperature on *A. flavus* growth, gene expression, and AFs production on other foods

Recently, there are some researches that reported the effect of a_w and temperature on the fungal growth and AFs production by *A. flavus* on other foods. In whole black peppercorns (*Piper nigrum* L.), the growth and AFs production of three *A. flavus* isolates and one *A. parasiticus* isolate were investigated using a full factorial design with seven a_w levels (0.826–0.984) and three temperatures (22, 30, and 37°C) (Yogendrarajah *et al.*, 2016). Among secondary models, the extended Gibson model was the best model to describe the combined effect of a_w and temperature on the growth rate of both fungal species in peppercorns. The highest population of *A. flavus* occurred at 0.92 a_w and 30°C, and the maximum yield of AFB_1 was also observed under this condition based on diverse secondary models. The estimated minimum a_w and temperature for the growth of *A. flavus* were 0.73–0.76 and 11–16°C, respectively. High variability in AFs production of different aflatoxigenic species limited the modelling of AFs production. Based on the research of Yogendrarajah *et al.* (2016), the limiting a_w and temperature should be considered to prevent the aflatoxigenic fungal species growth and AFs production in food during storage.

Peromingo *et al.* (2016) evaluated that the interaction between a_w (0.85, 0.90, and 0.95) and temperature (10, 15, 20, and 25°C) may occur on lag phases prior to growth, growth rates, and AFs production by two strains of each *A. parasiticus* and *A. flavus* on cured meat over 12 days. *Aspergillus flavus* CBS 573.65 had shorter lag phases than *A. parasiticus* CECT 2 688; however, the growth rates of the two strains were similar. The optimum growth and AFs production occurred at 0.95 a_w and 25°C. At 10°C and all tested a_w, no growth occurred for both species. Both species produced AFs when the a_w and temperature were $a_w \geq 0.90$ and $\geq 15°C$, respectively. Although similar AFB_1 production characteristics were found between the two species, the concentration of this toxin produced by *A. flavus* was much higher than that of *A. parasiticus*.

To elucidate the relationship between the relative expression of AFs-related genes and AFs production, Peromingo *et al.* (2017) evaluated the effect of different a_w and temperatures on the temporal relative expression of three genes in AFs biosynthesis cluster and their correlation with AFs production on dry-cured ham-based medium by *A. flavus* and *A. parasiticus*. In general, the expressions of the regulatory *aflR* and *aflS* genes were similar and much lower than the expression of the structural *aflP* gene. The expression of *aflR* and *aflS* genes in *A. flavus* increased over a decrease of a_w regardless of temperature. Regarding *A. parasiticus*, the highest and lowest expression values of both regulatory genes were observed at 0.95 a_w and 0.85 a_w, respectively. In contrast, the expression of *aflP* gene in both species was stimulated at low temperature and a_w. Furthermore, a strong correlation between the relative expression of *aflR* and *aflS* gene and AFs production was obtained under environmental conditions which simulate dry-cured ham ripening.

Effect of other environmental factors on *A. flavus* growth and AFs production on food

In addition to a_w and temperature, there are more environmental parameters that have been investigated for the influences on the growth and AFs production by *A. flavus* in many studies. It has been reported that pH, CO_2 level, and light treatment also exhibit significant effects on fungal growth and AFs production (Schmidt-Heydt *et al.*, 2008; Oms-Oliu *et al.*, 2010; Castellari *et al.*, 2015).

Regarding pH, Casquete *et al.* (2017) investigated the effect of pH (5.0, 5.5, and 6.0), a_w (0.90, 0.95, and 0.99), and temperature (15, 20, 25, and 30°C) on the lag phases, growth, and AFs production of three aflatoxigenic *A. flavus* strains (CQ7, CQ8, and CG103) on cheese-based medium. The results showed that the behaviour of *A. flavus* strains was affected by pH, a_w, and temperature; however, *A. flavus* growth was less affected by pH than the others. The CQ7 strain exhibited maximum growth at pH 5.5, 0.99 a_w, and 25°C. However, for the CQ8 and CQ103 strains, there was no difference between pH 5.5 and 6.0. The maximum AFs production on the cheese-based medium occurred at pH 5.0, 0.95 a_w, and 25 or 30°C, depending on the strain. Kosegarten *et al.* (2017) also indicated that an increase in pH value between 3.5 and 6.5 resulted in an increase in the growth of *A. flavus*.

For CO_2 level, Mousa *et al.* (2016) illustrated that the growth of *A. flavus* and AFs production was highly influenced by the CO_2 level (20%–80%) on paddy. In general, fungal growth rates and AFs production were negatively correlated with CO_2, whereas the lag phase durations were positively correlated with CO_2. However, the highest tested CO_2 level (80%) could not completely inhibit the fungi growth. Under 0.98 a_w, 20% and 80% CO_2 caused at least 59% and 88% reduction in growth and 47% and 97% reduction in AFs production, respectively. In addition, a significant inhibition of growth was observed at 75% CO_2 at both 0.95 and 0.92 a_w on agar medium. The population of *A. flavus* isolated from grains was inhibited at up to 75% CO_2 (Giorni *et al.*, 2008). Giorni *et al.* (2008) also has reported that the moisture maize treated with 25% CO_2 was sufficient to effectively reduce the development of *A. flavus*, but at least 50% CO_2 was required to significantly reduce the synthesis of AFs. Therefore, controlling pH and CO_2 levels during manufacturing and storage is an effective control strategy to avoid the contamination of *A. flavus* and subsequent AFs (Chulze, 2010).

Discussion and Conclusions

These previous studies have indicated that several environmental factors play important roles in regulating fungal growth, the expression of AF biosynthetic genes, and AFs production by *A. flavus* and *A. parasiticus*. Especially, a_w and temperature are limiting factors during storage (Liu *et al.*, 2017). Therefore, this review summarizes the different ranges of a_w and temperature and the optimal condition for *A. flavus* growth and AFs production on different food substrate
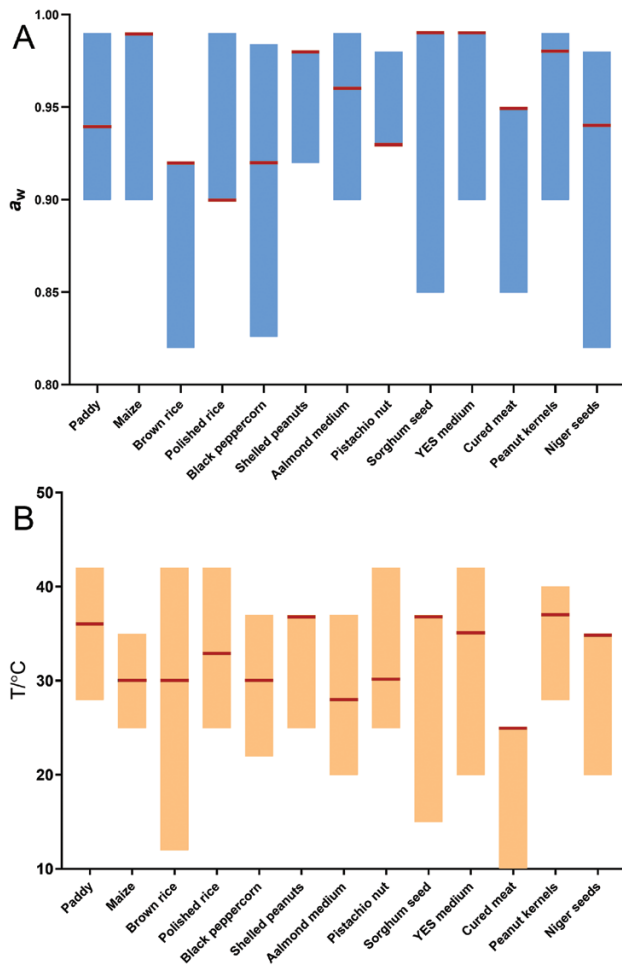
Figure 1. Comparison of a_w and temperature ranges and the optimal conditions (the black line) for the growth of *A. flavus* in different food matrices and formula medium. (A) *Aspergillus flavus* growth at different a_w. (B) *Aspergillus flavus* growth at different temperatures.

Figure 2. Comparison of a_w and temperature ranges and the optimal conditions (the black line) for the aflatoxin (AF) production in different food matrices and formula medium. (A) AF production at different a_w. (B) AF production at different temperatures.

and formula media (Figures 1 and 2). As shown in the two figures, the optimal conditions of $a_w \times$ temperature for *A. flavus* growth and AFs production vary on different foods or formula medium (Lv *et al.*, 2019). On yeast extract sucrose (YES) medium, the suitable temperatures for AFB_1 production were 25–30°C at 0.99 a_w, whereas the range changed to 30–35°C at 0.95 a_w (Abdel-Hadi *et al.*, 2012). On un-autoclaved peanut kernels, the suitable temperatures for AFB_1 production were 0.92–0.96 a_w and 25–33°C, and the highest level of AFB_1 was observed at 0.96 a_w and 28°C (Liu *et al.*, 2017). Similar findings were obtained on almond medium; the maximum fungal growth and AFB_1 production were observed at 0.96 a_w and 28°C (Gallo *et al.*, 2016). On un-autoclaved polished rice, high production of AFB_1 was observed at 0.90–0.99 a_w and 25–33°C, and the maximum amount of AFB_1 was obtained at 0.96 a_w and 33°C (Lv *et al.*, 2019). Moreover, these results suggest that the fungal growth and AFs production on foods can occur over a wider range of $a_w \times$ temperature than on formula medium. The diversity of optimal conditions may be due to the differences in media structure and nutrient availability (Ahmad *et al.*, 2013; Mousa *et al.*, 2013).

Compared with the investigations published before 2015, the recent publications pay more attention to the correlation between the expression of AFs biosynthetic genes and AFs production. The
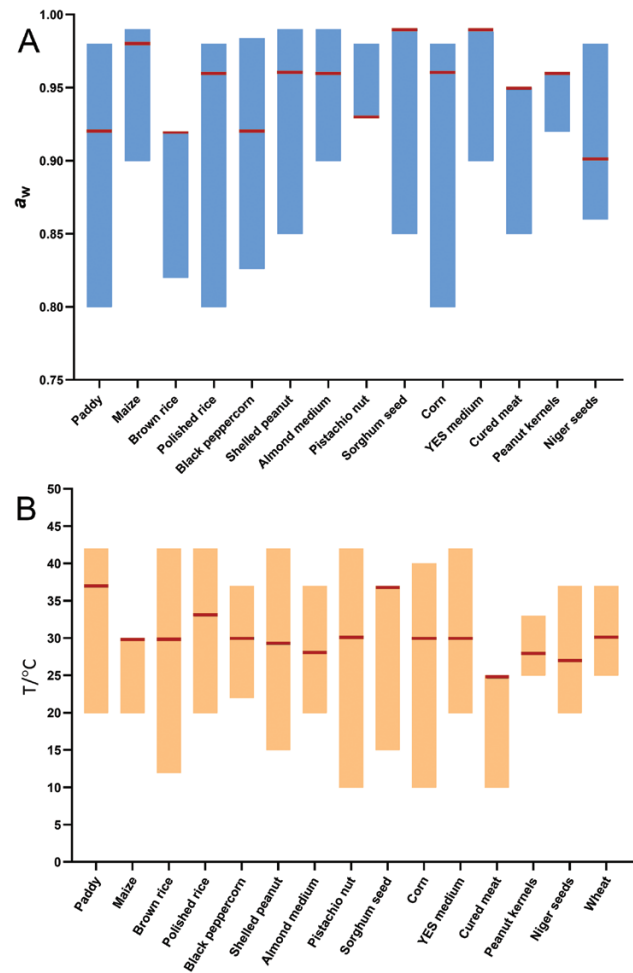
researchers attempt to get a good indicator of possible AFs contamination on foods by early detecting the expression of AFs-related genes. As the key regulatory genes in AFs biosynthesis gene cluster, *aflR* and *aflS* were highlighted. On YES medium, the down-regulation of *aflR* and *aflS* induced the inhibition of AFs production (Yu *et al.*, 2011). And the decrease in the expression ratio of *aflS/aflR* leads to transcription inactivation of AFs cluster. Similar findings were obtained on un-autoclaved peanut kernels (Liu *et al.*, 2017). At 42°C, the lower ratio of *aflS/aflR* resulted in lower AFB_1 production compared with 28 and 37°C.

In contrast, some literatures have reported that the *aflR* expression is not consistent with AFs production at several conditions. On a maize-based medium, the influences of a_w and temperature on AFB_1 production were different with the expression of *aflR* gene (Bernáldez *et al.*, 2017). On polished rice, the down-regulation of all the tested AFs structural genes at a_w 0.90 resulted in a low level of AFB_1 production compared with a_w 0.96, although *aflR* and *aflS* were both up-regulated (Lv *et al.*, 2019). Similar findings were reported by O'Brian *et al.* (2007), who obtained an opposite relationship between AFs biosynthesis and the expression of *aflR* and *aflS*. AFs production was completely inhibited at 37°C although both regulatory genes were highly expression in liquid A and M media.

Schmidt-Heydt *et al.* (2009) indicated that the transcription level of *aflS* was high at >37°C under almost all a_w ranges tested, but the amount of AFs production was low on YES medium. These results suggest that some other molecular mechanisms, such as post-transcriptional mechanism, were involved in modulating the transcriptional level of AFs structural genes and the subsequent AFs production.

Compared with the two regulatory genes *aflR* and *aflS*, the transcriptional levels of some structural genes seem to have more strong correlation to AFs production. Gallo *et al.* (2016) indicated that the effect of environmental factors on AFs biosynthesis had a better correlation to the transcriptional activation/inactivation of structural genes (*aflD* and *aflO*) than the two regulatory genes. Similarly, Abdel-Hadi *et al.* (2010) also obtained a good correlation between *aflD* expression and AFB_1 production by *A. flavus* in raw peanuts under different a_w levels. In addition, the significant differences between the relative expression of *aflD* at different temperatures (28, 37, and 42°C) were observed, resulting in the significant differences in AFB_1 production on peanut kernels (Liu *et al.*, 2017). On YES medium, the expression profile of *aflD*, *aflO*, and *aflP* was consistently correlated with the ability of AFs production (Scherm *et al.*, 2005). On polished rice, the structural genes (*aflM*, *aflN*, *aflO*, *aflP*, *aflQ*, *aflU*, and *nadA*) involved in the middle and late stages of AFs pathway from VERA (Versicolorin A) to AFB_1 were down-regulated at 37°C compared with 33°C under a_w 0.96 (Lv *et al.*, 2019). The lower expression of these structural genes led to the decrease of AFB_1 production. On peanuts kernels, a good positive correlation between the expression of *aflQ*, being involved in the final step of the AFs biosynthetic pathway and encoding an oxidoreductase, and AFB_1 level was observed under different a_w's and temperatures (Liu *et al.*, 2017). Taken together, these results suggest that an early detection of the expression of some key structural genes (*aflD*, *aflO*, *aflP*, or *aflQ*) can be a better indicator of possible AFs contamination on foods under different a_w's and temperatures compared with *aflS* and *aflR*.

Besides modulating a_w and temperature, modified atmosphere packaging is regarded as a promising food preservation technique because it integrates the control microbial activity and insects, which tend to maintain the quality of the products and extend the shelf life with minimal application of chemicals (Taniwaki *et al.*, 2009; Mousa *et al.*, 2016). CO_2 was proved to retard the fungal growth and inhibit AFs production by *A. flavus*. Taniwaki *et al.* (2009) indicated that no fungal growth of *A. flavus* was observed at 40% and 60% CO_2 when O_2 level was <0.5% while growth was observed on PDA and CYA at 20% CO_2. However, even at the lowest CO_2 level (20%) studied, the retarding effect of CO_2 on the growth rate of *A. flavus* was significant on paddy (Mousa *et al.*, 2016). Overall, 55%–100% reduction in fungal growth was achieved with CO_2 treatment. On moistened maize, modified atmospheres with 25%–50% CO_2 only contributed to 30%–35% reduction in *A. flavus* growth while as the concentrations of CO_2 increased up to 75%, the reduction of above 50% was obtained (Giorni *et al.*, 2008). The more reduction of fungal growth on paddy than maize with CO_2 treatment may be due to the more resistance of paddy's physical structure to the invading fungi (Mousa *et al.*, 2016).

Besides fungal growth, AFs production was also significantly inhibited by modified atmosphere with CO_2. Mousa *et al.* (2016) found that the reduction in AFs production on paddy with CO_2 treatment (20%–80%) was in the range of 65.8%–98.0%, 70.4%–94.6%, and 72.9%–96.8% at 0.98, 0.95, and 0.92 a_w, respectively. However, no significant reduction in AFs production on maize was observed with modified atmosphere enriched with 25% CO_2 and balanced with N_2 (Giorni *et al.*, 2008). With the application of 50%

and 75% CO_2, 46% and 58% overall reduction in AFs production was observed, respectively. Additionally, on PDA or CYA enclosed with a modified atmosphere with 20% CO_2 and <0.5% residual oxygen, no AFs production was observed for 30 days (Giorni *et al.*, 2008). It is important to notice that the residual O_2 may be a limiting factor for fungal growth and AFs production under modified atmosphere packaging (Taniwaki *et al.*, 2009).

The pH is also an important factor in determining *A. flavus* growth and AFs production. Klich (2007) indicated that the increase in pH (4–6) was related to the increase of *A. flavus* growth, and the expression of the AFs pathway gene was also affected by the pH of the medium. In some foods such as fermented food, salted food, and canned food, fungal growth and AFs production can be controlled by adjusting pH values. However, there are some limitations on the practical application of adjusting pH in most foods, especially the grains and spices listed in this paper.

In conclusion, the low a_w and temperature or the increase in the CO_2 level alone is not effective for complete control of *A. flavus* and AFs production. Integrating various post-harvest methods with synergistic functions may be more efficient for the complete inhibition of *A. flavus* growth and AFs production during storage and processing of foods. For example, reducing a_w is the prerequisite step to prevent *A. flavus* infection and AFs production, and controlling a_w and temperature and increasing the level of CO_2 in the atmosphere are useful strategies during storage. Good Agricultural Practices (GAP) and Good Processing Practices (GPP) represent primary preventive measures against *A. flavus* and the subsequent AFs. Integrating the two principles with the Hazard Analysis and Critical Control Point (HACCP) can be used efficiently (Mousa *et al.*, 2016). A better knowledge of the environmental factors governing fungal growth and AFs production provided in the above-mentioned recent researches would help in establishing optimal guidelines in GAP and GPP, preventive measures, and critical limits in HACCP plans.

Acknowledgements

Conflict of Interest

None declared.

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