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Research Article

Reduction of aflatoxin production by exposing *Aspergillus flavus* to CO_2

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Abstract: *Aspergillus flavus* is a saprophytic fungus contaminating different food and nut products by aflatoxin which is a major problem worldwide. Modified atmosphere packaging (MAP) could be an effective method for control of saprophytic fungi and their toxins and secondary metabolites production. This study gives the consequences of fungal isolates growing under MAP condition on potato dextrose agar (PDA). Two isolates of *A. flavus* (A42 and CHAO50) were packed under 100% CO_2, 100% O_2 and vacuum conditions. The mycelial growth of fungal isolates on PDA was controlled up to 80% with CO_2 treatment. Under the vacuum condition, mycelial growth of the isolates was inhibited up to 30%, while 100% oxygen had no inhibition on mycelial growth. Examination of isolates A42 and CHAO50 under UV light showed that both isolates produced yellow pigments on aflatoxin producing ability (APA) medium after 10 days. The levels of aflatoxin B_1, B_2, G_1, G_2 and total aflatoxin were analyzed chromatographically. The results revealed that the highest concentration of total aflatoxin was produced by fungal isolates grown in an atmosphere of 100% O_2, while the level of aflatoxins was significantly reduced in 100% CO_2.

Keywords: HPLC, modified atmosphere, aflatoxin, vacuum condition

Introduction

Aspergillus flavus is one of the important storage fungi producing aflatoxin which have carcinogenic potentials on human and animals. Aflatoxin contamination is a major problem worldwide, which reduces the quality of food and feed especially in storage conditions (Hell and Mutegi, 2011; Hassan *et al*., 2017). Fungal growth and production of secondary metabolites, such as aflatoxins in substrates, are dependent on moisture availability, favorable temperature and atmospheric conditions of storage (Shih and Marth, 1973). Thereby, application of modified atmosphere to prevent fungal growth is considered as an appropriate method to extend the useful life of some foods especially those with high relative humidity (Wolfe, 1980). Also, using a controlled atmosphere (CA) storage or modified atmosphere packaging (MAP) can increase the storage life of various products via decreasing respiration rate and controlling fungal and bacterial infections. The most common gases used for this purpose are: carbon monoxide, oxygen, nitrogen, carbon dioxide and sulfur dioxide (Taniwaki *et al*., 2001) Low oxygen concentrations (1-5 kPa) and high concentrations of carbon dioxide (5-10 kPa) in combination with storage at low temperature is considered as an optimal storage condition for fresh fruits and vegetables (Kader *et al*., 1989; Jacxsens *et al*., 1999).

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Exposure of some ochratoxigenic species to CO_2 (50%) showed that the growth of the fungal species including *Aspergillus ochraceus*, *Penicillium verrucosum* and *Aspergillus carbonarius* was inhibited by about 50-70% compared with the natural atmosphere (Cairns-Fuller *et al*., 2005). High concentrations of carbon dioxide and low oxygen levels during the *A. flavus* growth restrict aflatoxin production by the fungus (Taniwaki *et al*., 2010; Torres *et al*., 2014). Studies have demonstrated the inhibitory effect of carbon dioxide on *A. flavus* growth. For instance, under 25% CO_2, mycelia growth of *A. flavus* could effectively be prevented, but the lowest concentration of carbon dioxide for reduction of aflatoxin production was 50 % (Giorni *et al*., 2008). CO_2 concentrations around 60-80% reduce growth of the fungi such as *Rhizopus stolonifer*, *Curvularia lunata*, *Cladosporium* sp. and *Alternaira alternata* in stored rice seeds. However, control of *A. flavus* and aflatoxin production can be reached when the carbon dioxide is at the concentration of 80% (Gupta *et al*., 2014).

Since environmental conditions affect the growth and production of secondary metabolites in fungi, different treatments can be used to prevent fungal contamination during storage and packaging. The aim of this study was evaluation of CO_2, O_2 and vacuum effects on growth of *A. flavus* isolates and aflatoxin production.

Materials and Methods

Fungal isolates

Two isolates A42 and CHAO50 of *A. flavus* were provided by the culture collection of the Department of Entomology and Plant Pathology, Aburaihan Campus, University of Tehran, and Mycotoxins Research Laboratory of Iranian Research Institute of Plant Protection, respectively.

Incubation of fungal isolates in modified atmosphere

A plug cut from the leading edge of a 5-days-old culture of *A. flavus* on water agar was placed at the center of the plate containing Potato Dextrose Agar (PDA). Plates were incubated in glass jar containers under different conditions including: 1) Air as control, 2) Saturated concentration of carbon dioxide (100% CO_2), 3) Saturated concentration of oxygen (100% O_2), and 4) Vacuum condition with four replicates for each treatment, for 10 days. For this purpose, a vacuum was created for all treatments, then containers were evaluated with gas analyzer to be out of any gases, and targeted gases were injected. Percentage of mycelia growth was calculated using the formula $GMI\% = DC - \frac{DT}{DC} \times 100$ (Moslem and El-Kholie, 2009), where GM_I is the percentage of mycelial growth inhibition, DC is the colony diameter of control, and DT is the colony diameter of treatment.

Detection of aflatoxin production by *A. flavus in vitro*

For this evaluation, aflatoxin producing ability (APA) medium was prepared based on Hara *et al*. (1974) method, containing $(NH_4)H_2PO_4$ (10 g), K_2PO_4 (1 g), $MgSO_4$. $7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4$. $7H_2O$ (0.01 g), Sucrose (30 g), Corn steep liquor (0.5 g) and agar (20 g) per 1 liter distilled water, and then pH was adjusted to 5.5. An agar plug (containing mycelia) of every *A. flavus* isolates was placed at the center of plate containing APA medium and incubated at 25 °C. After 10 days dorsal surface of colonies were analyzed under UV light for the presence of yellow pigment (Razzagi abyaneh *et al*., 2000).

Aflatoxin analysis with HPLC

Aflatoxin extraction was performed based on Bragulat *et al*. (2001) method with some modifications. Four plugs of agar were sampled from the 7 days-old colonies grown on PDA and the amount of four grams PDA was dissolved in 20 ml of 80% methanol. The solution was shaken for 30 minutes on a shaker and filtered through Whatman filter paper No: 1. Filtered solutions were analyzed using reversed phase HPLC by Faroogh Life Sciences Research Laboratory.

The standard aflatoxin HPLC analysis and the eluting of samples were performed with a reversed phase HPLC with a waters EB 2695 pump, fluorescence detector 2475 and

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autosampler 2695, UV- vis detector 2487 and chromolite cell brand thenomenex. The analytical conditions were: H_2O/ACN/MeOH (6:2:3 v/v/v) mobile phase, 60-µl injection volume, 1 ml/min fluid speed for 20 min, excitation at 362 nm and emission at 425 for aflatoxins B1 and B2 and 450 nm for G1 and G2. The standard solution of aflatoxin mixtures included 1000 ng of aflatoxin B_1 and G_1 types, and 200 ng of dissolved B_2 and G_2 types per ml of methanol. Immunoaffinity column was used for aflatoxin B_1, B_2, G_1 and G_2 antibodies.

Statistical analysis

Experiments were conducted in a completely randomized design with four replications. Obtained data were analyzed using the SAS ver. 9.0 software. All data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test to delineate mean differences by the F values ($P < 0.01$) (Steel and Torrie, 1980).

Results

The effect of modified atmosphere on fungal growth

The results of this experiment showed that there are significant differences between different treatments at 1% level of probability. Percentage of mycelial growth inhibition for isolates A42 and CHAO50 was 81.83% and 84.43%, respectively, at the concentration of 100% CO_2 and it was 32.68% and 38.93%, respectively, at vacuum conditions. Oxygen concentration at 100% level had no inhibitory effect on mycelia growth in this experiment. The highest inhibition percentage of fungal mycelial growth was related to the saturation concentration of CO_2 (Fig. 1).

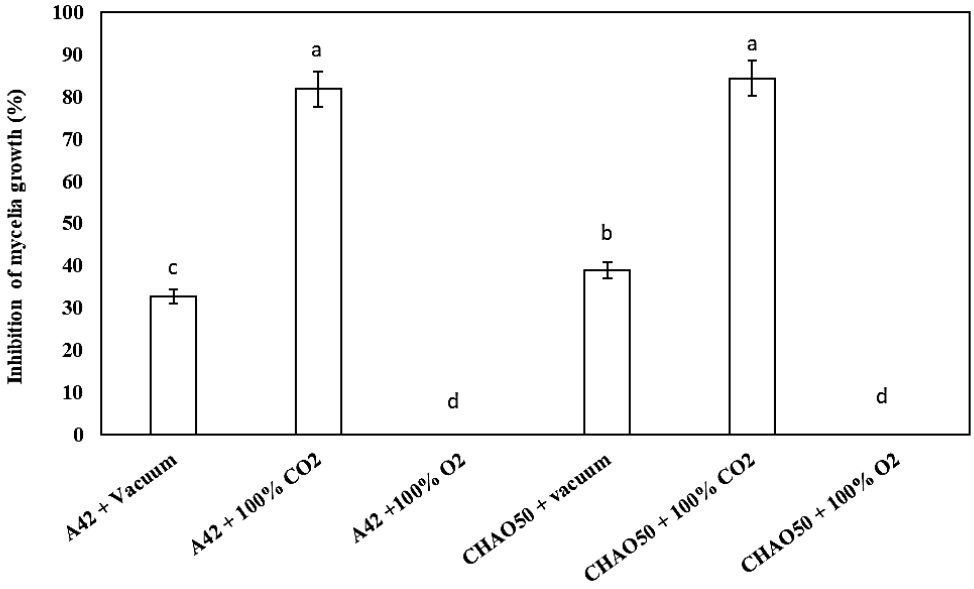

Figure 1 The inhibitory effect of different atmospheres on mycelia growth of *Aspergillus flavus* isolates A42 and CHAO50. Columns with the same letter are not significantly different at 1% level of probability (Duncan's multiple range). Data are the mean ± SE of four replicates.

Detection of fungal isolates toxinogenicity *in vitro*

Examination of isolates A42 and CHAO50 under UV light showed that both isolates produced yellow pigments on APA medium after 10 days (Fig. 2).

Effect of modified atmosphere on aflatoxin production

The results showed that the lowest level of aflatoxins B_1 (AFB_1) (36.8 µg/kg), B_2 (AFB_2) (2.2 µg/kg), G_1 (AFG_1) (ND: not detectable), G_2 (AFG_2) (ND) and total aflatoxin (AF_S) (39

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µg/kg) were produced by isolate A42 at the saturated concentration of CO_2 (Table 1). Isolate CHAO50 produced the lowest level of aflatoxins B_1 (360.67 µg/kg), G_1 (ND) and total aflatoxin (1357.67 µg/kg) at the saturated concentration of CO_2 (Table 1). But, the highest level of aflatoxins was produced by *A. flavus* isolates grown at the saturated O_2 condition (Table 1).

The level of aflatoxins produced by both isolates in vacuum condition was lower than control, while that was more than aflatoxins production of *A. flavus* isolates in 100% CO_2 condition (Table 1).

Figure 2 Production of yellow pigments by *Aspergillus flavus* isolates A42 (right) and CHAO50 (left) on APA medium after 10 days under UV light.

Table 1 Level of aflatoxins: B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2) and total aflatoxin (AF_S) production by *Aspergillus flavus* isolates A42 and CHAO50 in different modified atmosphere conditions.

Treatments	Level of aflatoxins (µg/kg)				
	B_1	B_2	G_1	G_2	Total AF_S
Control A2	153.32	ND	ND	ND	153.32
A42 + vacuum	48.12	9.27	12.30	ND	69.69
A42 + CO_2 100%	36.80	2.20	ND	ND	39.00
A42 + $O_2$100%	186.00	15.70	9.20	ND	210.90
Control CHAO50	23999.00	256.00	ND	242.00	24497.00
CHAO50 + Vacuum	4984.00	798.00	ND	ND	5782.00
CHAO50+ $CO_2$100%	360.67	723.00	ND	274.00	1357.67
CHAO50 + $O_2$100%	48118.00	927.00	123.00	ND	49168.00

ND = Not Detectable.

Discussion

The evaluation of the isolates A42 and CHAO50 growth revealed that the colony diameter after 10 days in the saturated CO_2 was considerably lower than control, while the colony diameter of isolates grown under saturated O_2 was equal to the control. In some similar studies, it has been shown that high concentrations of CO_2 prevent the fungal growth and it has been attributed to decreasing respiration rate (Taniwaki *et al*., 2001; Pateraki *et al*., 2007). Pateraki *et al.* (2007) suggested that 25% CO_2 could reduce the mycelia development of *Aspergillus carbonarius* and 50% CO_2 could inhibit the fungal growth completely regardless of the water activity (a_w). Water activity played an important role in reducing ochratoxin A (OTA) production by *A. carbonarius* and 93% a_w regardless of the concentration of CO_2, significantly reduces OTA production. As a result, the concentration of 50% CO_2 accompanied with 96% of a_w can reduce OTA production by *A. carbonarius* isolates (Pateraki *et al*., 2007). Moreover, postharvest use of modified atmosphere with 75% CO_2 regardless of levels of a_w reduced *A. flavus* mycelia growth up to 50% (Giorni *et al*., 2008) and another study on the *A. flavus* growth in cheese revealed that modified atmosphere with 40% CO_2 and 1% O_2 inhibits fungal growth about 45-65% (Taniwaki *et al*., 2001). The reduction of O_2 from 5% to 1% and 20% CO_2 had little effect on the production of ergosterol by *A. flavus* (Taniwaki *et al*., 2001). In another study, it was found that the growth of *A. flavus* in both PDA and CYA media in the atmosphere containing 80% carbon dioxide and 20% oxygen is very slow, and no aflatoxin was produced by fungi in both media (Taniwaki *et al*., 2010). Furthermore, Gupta *et al.* (2014) reported that 80% carbon dioxide concentration inhibits the incidence of *Aspergillus* species. Also, Mokble and Hashinaga (2004) showed that low concentration of CO_2 (2-20 kPa) had no inhibitory effect on the fungal growth, but high concentration of CO_2 (40-60%) inhibited the mycelial growth of all the studied fungi including *Alternaria alternata*, *Penicillium*

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expansum, *Rhizopus stolonifer*, *Geotrichum candidum*, *Colletotrichum acutatum*, *Botrytis cinerea*, *Fsuarium oxysporum* f.sp. *fragariae*, *Fusarium oxysporum* f.sp. *lycopersici*, *Glomerella cingulata*, *Phytophthora citrophthora*, *Monilinia fructicola*, *Penicillium italicum* and *Aspergillus niger*. In fact, the result of current study was consistent with these findings.

Results of this study showed that cultured isolates on APA produced the yellow pigment at the bottom of the colony under UV-light. Abbas *et al.* (2004) stated that the yellow pigment is secreted in the medium and it is easily visible on the back surface of the colonies grown on a semi- transparent agar medium such as PDA. Also, they reported that early screening of aflatoxin producer fungi by the yellow pigments is reliable and faster than other methods including blue fluorescent zones under long-wave UV light.

HPLC analysis showed that the level of total aflatoxins produced by isolates A42 and CHAO50 at 100% CO_2 concentration were decreased up to 75% and 77%, respectively. Gupta *et al*. (2014) reported 46% and 58% reduction in aflatoxin production in 50 and 75% of CO_2, respectively. On the contrary, the total aflatoxin production by isolates A42 and CHAO50 at 100% O_2 was increased by 37.5% and 100.7% compared with control. Paster *et al.* (1990) concluded that aflatoxin is produced in an aerobic process. Thereby, reducing oxygen and increasing CO_2 will prevent aflatoxin production. Also, Gupta *et al*. (2014) found that mycotoxins are produced at low CO_2 concentration and high levels of O_2, which is the suitable condition for fungal growth.

Taniwaki *et al.* (2010) noted that *A. flavus* on PDA does not produce aflatoxin under a modified atmosphere with 80% CO_2 and 20% O_2. In other research, Taniwaki *et al*. (2001) found that the level of aflatoxin B_1 production by *A. flavus* was reduced in the modified atmosphere with 20% CO_2 + 1% O_2 and 40% CO_2+ 5% O_2. So far the inhibition mechanism of the mycotoxins production by high concentrations of CO_2 and low levels of O_2 has not been cleared (Richard *et al*., 2004; Giorni *et al*., 2008).

HPLC analysis showed that total aflatoxin production by isolates A42 and CHAO50 in vacuum conditions was reduced to 54% and 76%, respectively. Scussel *et al.* (2011) in a study on the Brazil nuts, showed a significant reduction in the development of *A. flavus* by using adsorbent O_2 and O_3 with a vacuum. They suggested that the packaging of Brazil nuts with O_3 and vacuum is an alternative to reducing aflatoxin. Northolt and Bullerman (1982) found that controlled atmosphere (increasing the CO_2 content to 20% and reducing O_2 to about 2%) prevents the *A. flavus* growth and reduces the aflatoxin production by the fungus.

The results of the current study showed that the saturated concentration of CO_2 is efficient enough to control the growth of *A. flavus* isolates and their aflatoxin production. But, increasing oxygen concentration increased the level of aflatoxin production. Therefore, aflatoxin production can be reduced by decreasing the concentration of oxygen and/or increasing the CO_2 concentration. This study also showed that vacuum condition inhibited the growth and aflatoxin production by both isolates of *A. flavus*. There was a significant difference in aflatoxin production of the two fungal isolates, which could be attributed to the response of the isolates to atmospheric changes, which should be considered in subsequent studies.

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کاهش تولید آفلاتوکسین *Aspergillus flavus* در معرض CO_2

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چکیده: *Aspergillus flavus* یک قارچ ساپروفیت است که با تولید آفلاتوکسین باعث آلودگی مواد غذایی مختلف و محصولات خشکبار می‌شود و این یکی از مشکلات عمده در دنیا می‌باشد. بسته‌بندی با تغییر اتمسفر (MAP) به‌عنوان یک روش مؤثر برای کنترل قارچ‌های ساپروفیت و تولید توکسین‌ها و متابولیت‌های ثانویه آن‌ها می‌باشد. در این تحقیق، رشد جدایه‌های قارچی روی محیط‌کشت سیب‌زمینی-دکستروز-آگار (PDA) تحت شرایط اتمسفر تغییر یافته (MAP) مورد بررسی قرار گرفت. دو جدایه از قارچ *A. flavus* (A42 و CHAO50) تحت شرایط 100% CO_2، 100% O_2 و خلاء مورد بررسی قرار گرفتند. در شرایط 100% CO_2، رشد میسلیومی جدایه‌های قارچی روی محیط‌کشت PDA تا ۸۰٪ و تحت شرایط خلاء تا ۳۰ درصد کنترل شد. درحالی‌که 100% O_2 اثر بازدارندگی روی رشد میسلیومی جدایه‌های قارچی نشان نداد. بررسی جدایه‌های A42 و CHAO50 روی محیط کشت APA و زیر نور UV نشان داد هر دو جدایه بعد از ده روز، رنگدانه‌های زرد رنگ تولید می‌کنند. میزان آفلاتوکسین‌های B_1، B_2، G_1، G_2 و آفلاتوکسین کل به روش کروماتوگرافی مورد بررسی قرار گرفت. نتایج نشان دادند که بیش‌ترین میزان آفلاتوکسین توسط جدایه‌های قارچی در 100% O_2 تولید شده، درحالی‌که میزان آفلاتوکسین تولیدی در شرایط 100% CO_2 به‌طور معنی‌داری کاهش یافت.

واژگان کلیدی: اتمسفر تغییر یافته، افلاتوکسین، شرایط خلاء، HPLC

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