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Growth and aflatoxin production of *Aspergillus flavus* in fig-based medium: Effect of minerals and chelating agents

Aspergillus flavus'un incir bazlı besiyerinde gelişimi ve aflatoksin üretimi: Mineral maddelerin ve şelat ajanlarının etkisi

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Abstract

In this study, minerals required for the growth and aflatoxin production of A. flavus *were determined. Then, the effectiveness of some chelating agents such as citric acid, phytic acid and ethylenediaminetetraacetic acid (EDTA) in preventing mold growth and toxin production was investigated in a fig-based medium and in two synthetic media, called Czapek-dox agar (CZA) and potato dextrose agar (PDA).* A. flavus *did not grow in CZA without sodium nitrate during 6-day incubation at 30 °C. The absence of the other components (magnesium sulfate, iron sulfate, potassium chloride and potassium phosphate) did not significantly (p>0.05) affect the growth of the mold. However, the mold produced significantly (p<0.05) higher amounts of aflatoxins in the media without magnesium sulfate and iron sulfate.* A. flavus *grew faster on fig-based medium and produced higher amounts of aflatoxins in PDA. In all media tested in this study, EDTA was found to be more effective than the other agents in controlling the growth of* A. flavus*. It was also effective in inhibiting the production of aflatoxins. It was determined that 1.75 mM EDTA reduced aflatoxin production up to 97% in PDA. Citric and phytic acids could inhibit the growth of the mold only in PDA. All chelating agent reduced aflatoxin production over 90% in fig-based medium.*

Keywords: Template mycotoxin, mold, Ethylenediaminetetraacetic acid, Citric acid, Phytic acid, Aflatoxin, *Aspergillus flavus*.

Öz

Bu çalışmada, A. flavus *küfünün gelişmesi ve aflatoksin üretmesi için ihtiyaç duyduğu mineral maddeler tespit edilmiştir. Daha sonra, sitrik asit, fitik asit, etilendiamintetraasetik asit (EDTA) gibi bazı şelat ajanlarının; incir-bazlı bir ortam, Czapek-dox agar (CZA) ve patates dekstroz agar (PDA) besiyerlerinde küfün gelişmesi ve toksin üretmesini önlemedeki etkinliği araştırılmıştır. Sodyum nitrat içermeyen CZA besiyerinde 30 °C'deki 6 günlük inkübasyon süresince* A. flavus *küfünün hiç gelişmediği saptanmıştır. Diğer bileşenlerin (magnezyum sülfat, demir sülfat, potasyum klorür ve potasyum fosfatın) besiyerinde bulunmaması, küfün gelişimini istatistiksel olarak anlamlı bir şekilde etkilememiştir (p>0.05). Ancak magnezyum sülfat ve demir sülfat içermeyen besiyerlerinde istatistiksel olarak anlamlı düzeyde yüksek miktarlarda aflatoksin üretimi gözlenmiştir (p<0.05).* A. flavus *küfü, incir-bazlı ortamda daha hızlı gelişmiş ve PDA besiyerinde daha yüksek miktarda aflatoksin üretmiştir. Denenen tüm besiyerlerinde, EDTA,* A. flavus *gelişimini kontrol etmede diğer ajanlara göre daha etkili olmuştur. Ayrıca bu ajan, aflatoksin üretimini inhibe etmede de etkili bulunmuştur. Söz konusu ajanın 1.75 mM'lık konsantrasyonu, PDA besiyerinde aflatoksin üretimini %97'ye varan oranlarda azaltmıştır. Sitrik asit ve fitik asit küf gelişimini sadece PDA besiyerinde inhibe edebilmiştir. Denenen tüm şelat ajanları, incir-bazlı ortamda aflatoksin üretimini %90'ın üzerinde azaltmıştır.*

Anahtar kelimeler: Mikotoksin, küf, Etilendiamintetraasetik asit, Sitrik asit, Fitik asit, Aflatoksin, *Aspergillus flavus*.

1 Introduction

Mycotoxins are toxic metabolites of some mold species that can grow on food and feed. Aflatoxins are important mycotoxins produced by some toxigenic *Aspergillus* strains such as *A. flavus* and *A. parasiticus*. Many aflatoxin types have been identified but generally six of them, called aflatoxin B_1, B_2, G_1, G_2, M_1 and M_2 are regarded as potential food toxicants [1]. These toxins have mutagenic, teratogenic and carcinogenic effects on humans and animals. For instance, aflatoxin B_1 has been classified as "Group-I carcinogen" which means that there is sufficient evidence of carcinogenicity for this agent [2]. Due to their toxic properties, the permitted amounts of aflatoxins in various food products have been restricted by the legislation in many countries. These restrictions are generally based on the total aflatoxin content (sum of aflatoxins B_1, B_2, G_1, G_2) and the aflatoxin B_1 content alone, since it is the most toxic compound among others.

It is known that molds, like other microorganisms, depend on various factors such as energy source, water, oxygen, etc. for their growth. Minerals such as sodium, potassium, magnesium, etc. are also needed for various metabolic activities of the organism. Actually, molds have minimal growth requirements compared to other microorganisms. They may grow in a wide range of pH [3] and at low water activity levels [4].

Fig fruit is quite susceptible to the attacks of microorganisms including toxigenic molds. There are many critical steps in the course of dried fig production in terms of mold contamination and aflatoxin production. As a result, dried figs are among the commodities that are often reported due to the contamination with aflatoxins [5]-[7]. Aflatoxin contamination has become a chronic problem of the dried fig producer countries like Turkey, Greece, Spain, Italy, the US, etc. for decades. It constitutes not only an economic problem for the exporters but also a health risk for the consumers of the product.

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It is obvious that preventing mold contamination and aflatoxin production is the most reasonable solution to aflatoxin problem in dried figs as well as other commodities. There are quite a few studies that have demonstrated the antifungal effects of some chelating agents that are widely used in food applications. Citric acid and ethylenediaminetetraacetic acid (EDTA) have been recently tested for their antifungal potential [8]-[12]. Many factors such as treatment dose, temperature, fungal strain and the medium in which the cheating process takes place determine the effectiveness of the chelating agents in controlling mold growth and toxin production. Although dried figs are risky commodities in terms of aflatoxin contamination, as yet there are no studies examining the effectiveness of common chelating agents for controlling growth and aflatoxin production of toxigenic *Aspergillus* strains in figs or a fig-based medium.

The objective of the present study was to determine the mineral(s) required by toxigenic *A. flavus* for its growth and aflatoxin production. It was also aimed to evaluate the effectiveness of citric acid, phytic acid and EDTA in controlling the growth and aflatoxin production of the mold in different media including a fig-based medium.

2 Material and methods

2.1 Fungal strain and chemicals

A. flavus was chosen as the fungal species to be used in the study since it was previously shown as the dominant species in the fungal flora of the major fig production area in Turkey [13]. The toxigenic strain used in the study (MAM-200682) was obtained from Mold Culture Collection of the Scientific and Technological Research Council of Turkey (TUBITAK). The strain was maintained at -70 °C in 60% glycerol until use and activated on PDA at 30 °C for 7 d before the experiments.

The microbiological media used in the study (potato dextrose agar [PDA] and Czapek-dox agar [CZA]) were obtained from Merck (Darmstadt, Germany). Sodium nitrate ($NaNO_3$), sodium hydroxide (NaOH), disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) and nitric acid (HNO_3) were also purchased from Merck. Potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), sodium chloride (NaCl), potassium bromide (KBr), methanol and agar-agar were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Iron (III) sulfate pentahydrate ($Fe_2(SO_4)_3.5H_2O$) and ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Disodium EDTA (Na_2-EDTA) was obtained from BioShop (Burlington, Ontario, Canada). Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) and citric acid were from Scharlau (Barcelona, Spain). Phytic acid (50% in water) and sucrose were obtained from Acros Organics (Geel, Belgium) and Caisson Laboratories (Logan, UT, USA), respectively.

2.2 Preparation of modified media and measurement of mycelial growth

In order to investigate the effects of minerals on growth and aflatoxin production of *A. flavus*, CZA, which contains all the minerals as separate ingredients, was chosen as a model medium. Originally, the composition of CZA was (for 100 mL): 3 g sucrose, 0.3 g $NaNO_3$, 0.1 g $MgSO_4.7H_2O$, 0.0012 g $Fe_2(SO_4)_3.5H_2O$, 0.05 g KCl, 0.125 g KH_2PO_4 and 1.3 g agar-agar [14]. In order to prepare the modified media, aqueous stock solutions of $NaNO_3$, $MgSO_4.7H_2O$, $Fe_2(SO_4)_3.5H_2O$, KCl and KH_2PO_4 were prepared.

It is known that *Aspergillus* spp. use $NaNO_3$, $MgSO_4.7H_2O$, $Fe_2(SO_4)_3.5H_2O$ as sources of sodium, magnesium and iron, respectively [15],[16] and therefore modified media were prepared by omitting one of these ingredients. In the case for potassium, two ingredients (KCl and KH_2PO_4) were tested. A detailed description of the modified media prepared is given in Table 1. All the media were prepared in borosilicate glass bottles, sterilized by autoclaving for 15 min at 121 °C, cooled and then poured into plastic petri dishes, 85 mm in diameter. Five replicate plates were used for each modification.

Table 1. Detailed description of modified media prepared.

Modification	Content of the modified media
None (control)	sucrose, $NaNO_3$, $MgSO_4.7H_2O$, $Fe_2(SO_4)_3.5H_2O$, KCl, KH_2PO_4, agar-agar and distilled water
Omitting $NaNO_3$	sucrose, $MgSO_4.7H_2O$, $Fe_2(SO_4)_3.5H_2O$, KCl, KH_2PO_4, agar-agar and distilled water
Omitting $MgSO_4.7H_2O$	sucrose, $NaNO_3$, $Fe_2(SO_4)_3.5H_2O$, KCl, KH_2PO_4, agar-agar and distilled water
Omitting $Fe_2(SO_4)_3.5H_2O$	sucrose, $NaNO_3$, $MgSO_4.7H_2O$, KCl, KH_2PO_4, agar-agar and distilled water
Omitting KCl	sucrose, $NaNO_3$, $MgSO_4.7H_2O$, $Fe_2(SO_4)_3.5H_2O$, KH_2PO_4, agar-agar and distilled water
Omitting KH_2PO_4	sucrose, $NaNO_3$, $MgSO_4.7H_2O$, $Fe_2(SO_4)_3.5H_2O$, KCl, agar-agar and distilled water

The radial growth of *A. flavus* in different media during incubation was measured using a method suggested by Karaca et al. [17]. Briefly, the center of the medium in the petri dish was inoculated with mycelial plugs which were extracted using the back end of a sterilized Pasteur pipette (5 mm in diameter) from 7-day-old *A. flavus* cultures. Then, the dishes were incubated at 30 °C for up to 6 days. The radial mycelial growth (mm) in each plate was determined on the 2nd, 4th and 6th days of incubation. Fungal colony diameters were measured vertically and horizontally and the averages were calculated.

2.3 Assessment of mycelial growth and aflatoxin production of *A. flavus* in different media

To determine the most appropriate medium for growth and aflatoxin production of *A. flavus*, experiments were conducted on fig-extract agar, CZA and PDA.

Fig-extract agar was prepared based on the formulation of PDA medium. Originally, the composition of the PDA medium consists of potato infusion (200 g), dextrose (20 g), agar-agar (20 g) and distilled water (1 L) [18]. It is obvious that the minerals required for the growth of the mold are provided from the potato infusion in this medium. For the preparation of fig-extract agar, dried fig extract was used instead of the potato infusion in the formulation of PDA.

Aflatoxin- and additive-free dried figs were kindly supplied from a local market in Denizli, Turkey, cut into small pieces and boiled for 30 min in 1 L of distilled water in a pot. The amount of dried figs to be used for boiling was determined according to the results of a preliminary experiment conducted to test different amounts (150, 200, 250 g) of dried figs. Since the

amount of dried figs used in this step did not affect the growth of the mold, we decided to use 200 g of dried figs, the equivalent amount of potatoes in the PDA formulation. After boiling, the content of the pot was filtered through cheesecloth and 20 g of agar-agar (the same amount in PDA) was added. Since the sugar content of the dried figs is quite high [19], no additional sugar (dextrose or sucrose) was added to the medium. The medium, which was prepared as described above, was sterilized (121 °C, 15 min), poured into petri dishes and cooled to room temperature. Flow chart for preparation of fig-extract agar medium is given in Figure 1. The fig-extract agar medium, also PDA and CZA, were inoculated and incubated as described above and the radial mycelial growth was measured periodically during incubation. Aflatoxin analyses were conducted at the end of incubation period.

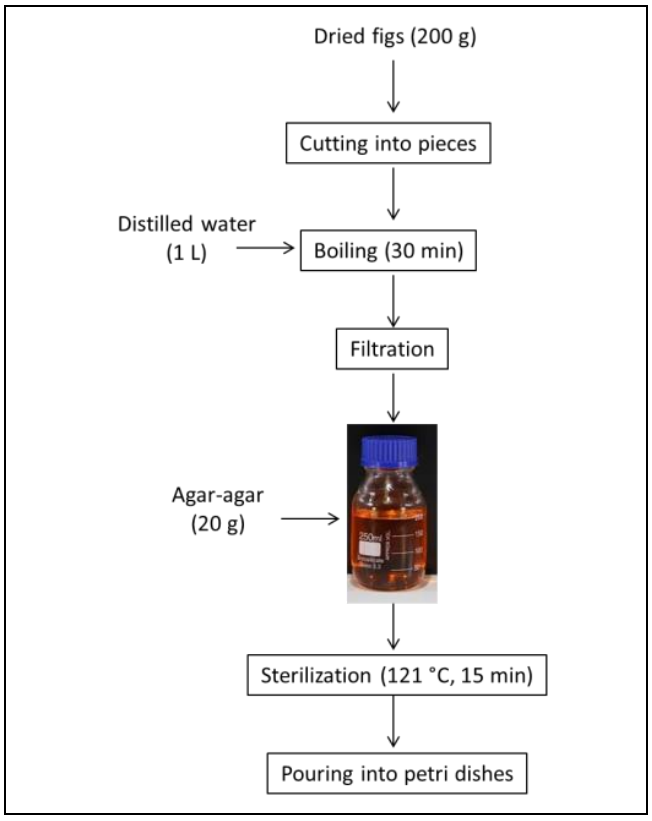


Figure 1. Flow chart for preparation of fig-extract agar medium.

2.4 Assessment of the effects of chelating agents on mycelial growth and aflatoxin production of *A. flavus* in different media

Efficacy of chelating agents in controlling growth and aflatoxin production of *A. flavus* in different media was studied. The three media used for this purpose were CZA, PDA and fig-extract agar. The agents (citric acid, phytic acid and EDTA) tested in this experiment were selected as they are commonly used for chelating purposes in food applications.

Determination of the concentrations of the chelating agents was based on the concentration of $NaNO_3$ in CZA as it was the most abundantly found compound in this medium. The concentration of this compound was calculated in millimolar (mM) and, 0.025 and 0.25 fold of this value were determined as the minimum and maximum concentrations, respectively, of the chelating agents to be tested in the experiments. Accordingly, 0.175, 0.875 and 1.75 mM of citric acid and EDTA were tested. Since a solidification problem was observed when 1.75 mM phytic acid was added to the medium, the concentrations to be tested for this agent were determined as 0.175, 0.438 and 0.875 mM. Stock solutions of the chelating agents were prepared and appropriate volumes of these solutions (corresponding concentrations) were added to each medium. Media without chelating agents served as controls.

A. flavus growth in different media with or without chelating agents was monitored during incubation according to the procedure described above. Five replicate plates were used for each chelating agent and the agent concentration. The results were expressed as the percentage of radial mycelial growth inhibition according to the formula: (dc-dt) / dc x 100. Here, dc is the average diameter of the fungal colony on control plates and dt is the average diameter of the fungal colony on chelating agent-amended plates. Aflatoxin analyses were conducted at the end of incubation period.

2.5 Analysis of aflatoxins

In the literature, it was reported that *A. flavus* produced the maximum amount of aflatoxins within 5-7 days at 30 °C [20]. After conducting experiments with *A. flavus* contaminated peanuts, Ellis et al. [21] reported that maximum toxin production occurred after 21 days of storage at 25 °C. In the light of these data, all aflatoxin analyses in the present study were carried out after an incubation period of 21 days at 30 °C.

Aflatoxins in the samples were determined according to official procedures (AOAC 999.07) with slight modifications [22]. Briefly, the content of the petri dish (approximately 20 g) was transferred to a Waring blender and 2.5 g of sodium chloride, 7.5 mL of distilled water and 70 mL of methanol were added. After blending for 1 min, the content of the blender was filtered through coarse filter paper. A portion of the filtrate (5 mL) was diluted with 10 mL of phosphate buffer solution (PBS). The PBS had been previously prepared by solving 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.45 g Na_2HPO_4 in 1000 mL distilled water and adjusted to pH 7.4 with NaOH.

The diluted extract (15 mL) was applied to immunoaffinity column (Aflatest, Vicam Watertown, MA, USA) at a steady state flow rate of approximately 3 mL/min. Then, the column was washed with distilled water (10 mL) twice at a flow rate of 5 mL/min. After passing air through the column with a syringe for several times, the aflatoxins were eluted by applying 1 mL of high pressure liquid chromatography (HPLC) grade methanol and 1 mL of bidistilled water. The eluate was collected in an amber vial and used in HPLC analysis.

Analysis of aflatoxins was carried out using an HPLC system (Shimadzu LC-20AD, Kyoto, Japan) consisting of a pump (Shimadzu LC-20A), a degasser (Shimadzu DGU-20A), a column oven (Shimadzu CTO-20A) and a fluorescence detector (Shimadzu RF-20A). The column oven was set at 25 °C and the fluorescence detector was set at an excitation wavelength of 360 nm and an emission wavelength of 430 nm. The analytical column was ODS 2 Hypersil (3 µm, 150x4.6 mm I.D., Thermo Scientific, Waltham, MA, USA). A post-column derivatization was employed using an electrochemical derivatization device (Coring System Diagnostix GmbH, Gernsheim, Germany). The mobile phase was methanol:water (40:60, v/v) + 216 mg KBr + 636 µL 4 M HNO_3 at a constant flow rate of 1 mL/min. A loop with 20 µL volume was used for sample injections.

A series of standard solutions were prepared from a mixed standard solution of aflatoxins (Supelco, Bellefonte, PA, USA; containing 1, 0.29, 0.99 and 0.27 µg of aflatoxins B_1, B_2, G_1 and G_2, respectively, in 1 mL of methanol) by appropriate dilutions in methanol. These solutions were used to prepare five-point calibration curves with concentrations of 2-10, 0.58-2.9, 1.98-9.9 and 0.54-2.7 µg/L aflatoxins B_1, B_2, G_1 and G_2, respectively. These calibration curves were used to quantify aflatoxins produced in the media. Triplicate injections were made for each concentration. Other calibration curves were prepared using low concentrations of aflatoxins to determine the limit of detection (LOD) and the limit of quantification (LOQ) of the method. These curves covered the ranges of 0.025-0.5, 0.0075-0.145, 0.2475-0.495 and 0.00675-0.135 µg/L for aflatoxins B_1, B_2, G_1 and G_2, respectively. LOD and LOQ values were calculated by the calibration curves and the following equations:

$$LOD = \frac{3.3 \times SD}{m} \quad (1)$$

$$LOQ = \frac{10 \times SD}{m} \quad (2)$$

Here, SD is the standard deviation of the response and m is the slope of the calibration curve. LOD and LOQ values were determined as 0.03 and 0.1 µg/L for aflatoxin B_1, 0.005 and 0.01 µg/L for aflatoxin B_2, 0.02 and 0.07 µg/L for aflatoxin G_1 and 0.01 and 0.03 µg/L for aflatoxin G_2, respectively. For recovery tests, medium samples were spiked with standard solution of aflatoxins (to a final aflatoxin B_1 concentration of 1 ng/g). The samples were let to settle for 15 min and then the extraction and injection procedures were conducted as described above. Triplicate injections were made and recovery values were calculated. The average recovery values for aflatoxins B_1, B_2, G_1 and G_2 were 87%, 60%, 120% and 85%, respectively. The results given in the present study were corrected for the average recovery values.

2.6 pH measurement

The pH values of all the media used in this study were measured with a bench-top pH-meter (HI 2211 pH/ORP Meter, HANNA Instruments, USA). Three measurements were recorded for each medium.

2.7 Statistical analysis

Statistical evaluations were accomplished using analysis of variance (ANOVA) techniques with the help of a statistical package program (Minitab v. 16, Minitab Inc., State College, PA, USA). Significant differences among means were identified by Tukey's mean test ($p<0.05$).

3 Results and discussion

3.1 The effect of the absence of minerals on growth of *A. flavus*

The effects of the absence of minerals on the growth of *A. flavus* during 6 d incubation at 30 °C are shown in Figure 2. Mean colony diameters measured in control plates on the 2nd, 4th and 6th days of incubation were 22.80±7.08, 50.88±4.92 and 75.80±4.50 mm, respectively. In the modified media prepared without $MgSO_4$, $Fe_2(SO_4)_3$, KCl or KH_2PO_4, these values were 18.20-20.00, 46.20-49.00 and 75.80-76.00 mm, respectively. On the other hand, no growth was observed throughout the 6 d incubation period in the medium prepared without $NaNO_3$. There were no significant differences among the growth rates determined in control plates and in the modified media ($p>0.05$) with the exception of the one without $NaNO_3$. Therefore, it can be concluded that Mg, Fe and K did not have any positive or negative effect on the growth of *A. flavus* at concentrations found in medium formulation. Whereas early studies stated that the growth of *A. flavus* and *A. parasiticus* were stimulated by the presence of Mg [23], Fe [24] and K [25]. On the contrary, these minerals and/or their salts were reported to have an inhibitory effect on mold growth above a certain concentration [26]-[28]. But, at low concentrations (i.e. those naturally found in fruits), minerals generally stimulated the growth of some important toxigenic fungi such as *Aspergillus* [29], *Penicillium* [30] and *Fusarium* [31].

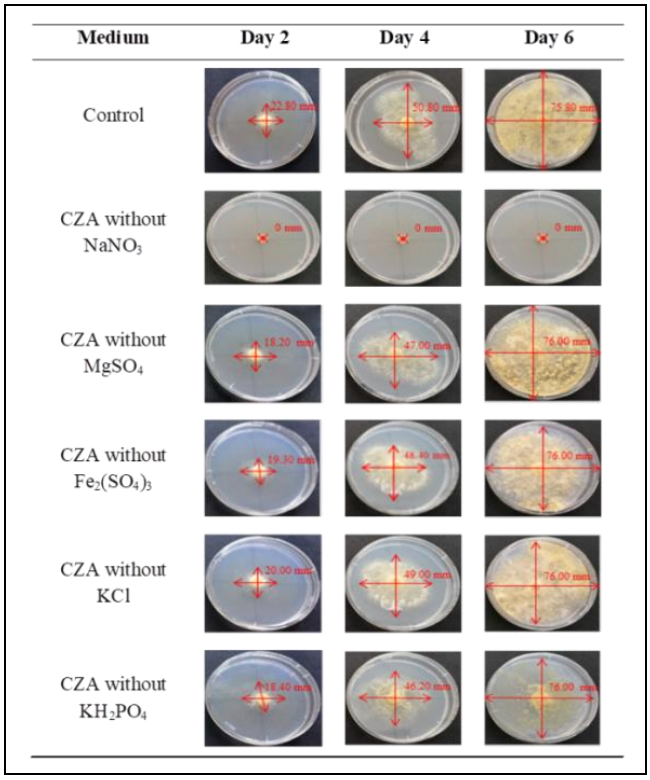


Figure 2. The effect of the absence of the minerals on the radial mycelial growth of *A. flavus* during 6 d incubation at 30 °C.

Another important result obtained in the present study is that *A. flavus* did not grow in the medium prepared without $NaNO_3$ throughout the 6-d incubation period. To clarify the cause of this observation; different sources of sodium and nitrate, Na_2SO_4 and KNO_3, respectively, were included separately in the formulation of the media. After the incubation period; there was no difference between the growth rates determined in control plates and in the medium with KNO_3 and no growth was observed in the medium prepared with Na_2SO_4 (data not shown). These results indicate the dependency of mold growth on nitrate and that *A. flavus* uses $NaNO_3$ as a nitrogen source rather than a sodium source. Various compounds such as amino acids, nitrate, nitrite, urea, ammonia, ammonium nitrate, ammonium sulfate were reported to be used as nitrogen source by *A. flavus* for metabolic activities [32]-[34]. Mehl and Cotty [35] examined the growth of eight genetically distinct *A. flavus* isolates in modified CZA media with varying concentrations of sucrose (8.8-88 mM) and $NaNO_3$ (3.5-35 mM), which are the only sources of carbon and nitrogen in the medium, respectively. The authors reported that the competition

between paired isolates was affected by varying concentrations of carbon and nitrogen. McAlpin and Wicklow [36] examined the growth of *Petromyces alliaceus*, a fungus classified in *Aspergillus* section *Flavi*, on modified CZA media and observed that a greater percentage of the stromata formed ascocarps when the $NaNO_3$ in CZA was replaced with an equivalent amount of available nitrogen supplied by ammonium tartrate, glutamic acid, or serine. Most recently, Wang et al. [37] showed that glutamine amino acid is the optimal nitrogen source for aflatoxin biosynthesis in *A. flavus* grown in CZA medium.

3.2 The effects of different media on growth of *A. flavus*

The effect of medium type on growth of *A. flavus* during 6 d incubation at 30 °C can be seen in Table 2. On the second day of incubation, the growth of *A. flavus* was significantly slower in CZA than those in the other media ($p<0.05$). However, on the following days, the difference between the mold grow rates in CZA and PDA disappeared ($p>0.05$). Despite the insignificant difference between the growth rates in PDA and fig-extract agar on the last sampling day, *A. flavus* grew faster in fig-extract agar than the other media throughout the incubation. It shows that fig-extract agar is a more suitable medium for *A. flavus* growth than CZA and PDA. After examining a plenty of fruits, Morton et al. [38] indicated that fig serves as a good substrate for *A. flavus* and *A. parasiticus* growth due to its high sugar and rich vitamin and mineral contents. Ikechi-Nwogu and Elenwo [39] inoculated spores of many fungal species (including *A. flavus*) into potato dextrose and groundnut dextrose broth and incubated at 27 °C for 7 d. They reported that groundnut dextrose broth supported the growth of all fungi tested better than potato dextrose broth because the former contains more vitamins and minerals vital to fungal growth.

Our results revealed that the growth rate of *A. flavus* varies remarkably on different media. Obviously, it is due to the differences in the composition of different media. Cotty [40] compared four media (*Aspergillus flavus/parasiticus* agar, rose bengal agar, modified rose bengal agar and CZA) for the isolation of *A. flavus* group fungi. According to the author, modified rose bengal agar is the most useful medium for studying population biology since it does not contain any complex carbon and nitrogen sources.

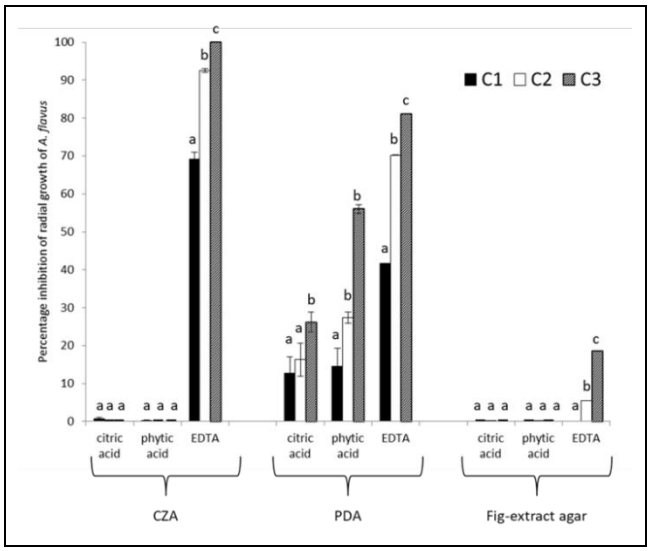
Table 2. Radial mycelial growth (mm) of *A. flavus* on CZA, PDA and fig-extract agar during 6-day incubation at 30 °C. [a, b].

Medium	Day 2	Day 4	Day 6
CZA	16.80±1.48 c	43.40±0.89 b	74.00±1.46 b
PDA	21.40±1.82 b	50.00±8,46 b	74.80±1.30 ab
Fig-extract agar	32.20±1.10 a	74.90±1.24 a	76.00±0.01 a

[a]: For each sampling day, mean values with different letters are significantly different according to Tukey's Multiple Range Test ($p<0.05$).
[b]: Data represent mean values and standard errors of five replicates.

3.3 The effects of chelating agents on growth of *A. flavus*

The effects of chelating agents on radial growth of *A. flavus* in different media after incubation at 30 °C are shown in Figure 3. As can be seen from the figure, citric acid and phytic acid failed to control the growth of *A. flavus* in CZA and fig-extract agar. There were no significant differences among fungal growth rates observed in the presence and absence of these agents in CZA and fig-extract agar media ($p>0.05$). In parallel to our results, Schultz and Muller [41] reported that citric acid could not inhibit the growth of any molds and yeasts in feedstuffs. However, there are also a number of studies that report the inhibition potential of both citric acid [42],[43] and phytic acid [44],[45].



[a]: C1, C2 and C3 were 0.175, 0.875 and 1.75 mM for citric acid and EDTA and 0.175, 0.438 and 0.875 mM for phytic acid. Data represent mean values and standard errors of five replicates. For a specific chelating agent tested in a specific medium, values with different letters are significantly different according to Tukey's Multiple Range Test ($p<0.05$).

Figure 3. The effects of citric acid, phytic acid and EDTA on the radial growth of *A. flavus* on CZA, PDA and fig-extract agar at the end of incubation at 30 °C for 6 d. [a]

According to our results, PDA was the only medium in which citric acid and phytic acid could inhibit the growth of *A. flavus*. In this medium, inhibition rates of 12.7-26.2% and 14.6-56.0% were recorded in the presence of citric acid and phytic acid, respectively. Inhibition rates increased as the concentration of the agents increased and the maximum inhibition rates were recorded in the presence of maximum concentrations of both citric acid (1.75 mM) and phytic acid (0.875 mM). Similar inhibition rates were observed by other authors on PDA amended with citric acid. Askarne et al. [46] reported that the growth of *Penicillium italicum* was inhibited by 29.2 and 55.2% in PDA amended with 0.02 and 0.2 M citric acid, respectively. Gowda et al. [47] recorded inhibition rates of 36-64% against *A. parasiticus* in PDA with citric acid. In addition, phytic acid was reported to be effective against *Botrytis cinerea* [48] in PDA and against *Penicillium expansum* [49] when used in combination with *P. caribbica* yeast. According to our results, citric acid and phytic acid inhibited the growth of *A. flavus* in PDA but were not effective against the same mold in two of three media (CZA and fig-extract agar) tested in this study. These results show that antifungal potentials of these chelating agents are strongly related to the content and distribution of the minerals of the medium where the mold grows. In our case, most of the compounds present in CZA and fig-extract media could not be chelated by citric acid or phytic acid. On the other hand, some important compounds required for the growth of *A. flavus* present in PDA were successfully chelated by the same agents.

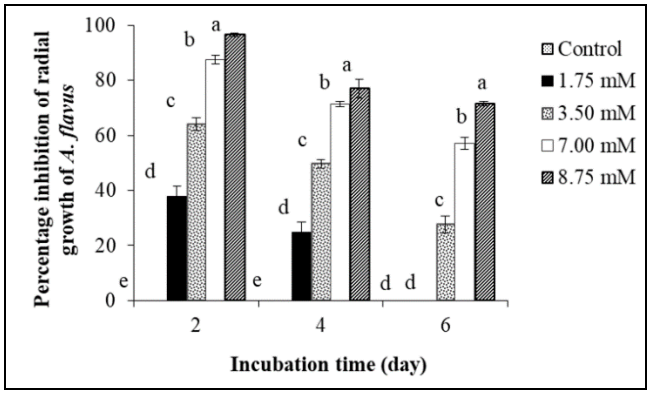
Among the chelating agents tested in the present study, EDTA was the most effective one against *A. flavus*. This agent inhibited *A. flavus* growth at varying degrees in all media tested. Inhibition rates recorded in CZA, PDA and fig-extract agar were 69.2-100, 41.7-81.0 and 0-18.6%, respectively. Inhibition rates

significantly increased as the concentration of EDTA increased in all media tested ($p<0.05$). A complete inhibition of mold growth was observed throughout the 6 d incubation period in CZA containing EDTA at 1.75 mM. Abrunhosa and Venancio [50] tested different doses of Na_2-EDTA (0.1-10 mM) against *A. carbonarius*, *A. ibericus*, toxigenic and non-toxigenic strains of *A. niger* on Czapek yeast extract agar. The authors observed dose-dependent inhibitions of all molds and recorded a maximum inhibition rate of 89%. De Lucca [51] reported that EDTA (0-1600 µg/mL) added to PDA inhibited the growth of various mold strains including *A. flavus*.

As mentioned before, *A. flavus* did not grow at all in CZA medium prepared without $NaNO_3$ throughout the 6 d incubation period. We proposed that $NaNO_3$ is absolutely necessary for mold since it is the only nitrogen source in CZA. EDTA could strongly inhibit the growth of *A. flavus* in CZA and a 100% inhibition was achieved when it is used at the highest concentration (1.75 mM) tested in our assays. To explain this situation, a chelating process that takes place between EDTA and nitrate can be proposed. Thus, $NaNO_3$ (the only nitrogen source found in the medium) might be transformed into an unavailable form for metabolic activities of the mold. However, this is unlikely to occur because both compounds (nitrate and EDTA) are negatively charged. An alternative mechanism is that EDTA could have a role in denitrification process and reduce nitrate to some antimicrobial compounds that are potentially effective against *A. flavus*. Denitrification can be defined as reduction of nitrate to nitrite, nitric oxide, nitrous oxide and nitrogen under aerobic or anaerobic conditions [52]. Maas et al. [53] reported that Fe (II) EDTA plays an important role in the biological denitrification process as a suitable electron donor. Nitric oxide, one of the main products of denitrification process, was shown to inhibit the growth of *A. niger*, *P. italicum* and *Monilinia fructicola* under *in vitro* conditions [54]. Nitrite, another potential product of denitrification, is a well-known antimicrobial compound which is effective against many organisms including molds [55]. Therefore, EDTA could start up a reaction -or a series of reactions- that result in the reduction of nitrate to some compounds with antimicrobial properties (nitrite, nitric oxide, etc.) and these compounds could be primarily responsible for the observed antifungal effect against *A. flavus*.

It can be clearly seen from Figure 3 that the most limited inhibitory effect of EDTA against *A. flavus* was in fig-extract agar. When used at the minimum tested concentration (0.175 mM), even no inhibition was observed. In order to see if higher inhibition rates would be obtained in the food medium, we decided to test increased concentrations of EDTA in fig-extract agar. However, due to the low solubility of EDTA, Na_2-EDTA was used in our following experiments.

Figure 4 shows the effect of different concentrations (1.75-8.75 mM) of Na_2-EDTA on radial growth of *A. flavus* in fig-extract agar during 6 d incubation at 30 °C. Na_2-EDTA was found to be significantly effective in controlling the growth of *A. flavus* in fig-extract agar ($p<0.05$). Up to 96.6% inhibition was achieved when this agent was used at the maximum concentration tested (8.75 mM). Inhibitory effect was relatively high at the beginning and gradually decreased during the rest of the incubation period at all Na_2-EDTA concentrations tested. One explanation for this observation could be that Na_2-EDTA extended the lag phase (adaptation to conditions) and thus delayed the onset of log phase (exponential growth) of *A. flavus*. Another reason could be that chelating ability of Na_2-EDTA diminished with time and the required substances for growth (minerals, etc.) became more available in the medium. In addition, inhibition rates obtained with Na_2-EDTA were higher than those obtained with EDTA when used at the same concentration. For instance; at 1.75 mM, the use of Na_2-EDTA resulted in 24.7% inhibition whereas EDTA caused 18.6%. These results show that Na_2-EDTA was more effective than EDTA in controlling *A. flavus* growth under *in vitro* conditions. A similar conclusion was drawn by Basaran [42] after her survey on *A. parasiticus*.



[a]: Data represent mean values and standard errors of five replicates. For each sampling day, mean values with different letters are significantly different according to Tukey's Multiple Range Test ($p<0.05$).

Figure 4. The effect of different concentrations (1.75-8.75 mM) of Na_2-EDTA on radial growth of *A. flavus* in fig-extract agar during 6 d incubation at 30 °C. [a]

3.4 The effects of chelating agents on the pH values of the media

It was reported that a pH value in the range of 3.4-10.0 is required for the growth of toxigenic *A. flavus* [56]. Holmquist et al. [26] reported that maximum growth of *A. flavus* and *A. parasiticus* were observed at pH 5 in corn and in Sabouraud Dextrose Agar. The pH values of the media could possibly be affected by the addition of chelating agents and pH is one of the determinative factors for mold growth. Moreover, chelating abilities of the agents could vary depending on the pH of the medium. For instance, enhanced chelating abilities were reported at low pH values for citric acid and at high pH values for EDTA [57]. Therefore, the pH values of the media amended with chelating agents were measured. The results are given in Table 3.

All the agents added to the media caused decreases in pH values. As the amounts of the agents added to the media were increased, the decreases in pH were more pronounced. It was also the case for EDTA which was found to be effective in inhibiting the growth of *A. flavus* in all media tested in a dose-dependent manner. However, EDTA is known to be more efficient in chelating minerals at high pH values [57]. Therefore, alternative mechanisms other than chelating could be responsible for the success of EDTA in controlling growth of the mold. The minimum pH values were 4.47 and 3.82 which were measured in PDA amended with citric acid and phytic acid, respectively. PDA was the only media in which citric acid and phytic acid could inhibit *A. flavus* growth. In the light of these findings, two possible mechanisms could be proposed to explain the inhibition effectiveness of these agents. First, citric acid and phytic acid could form strong chelates with the substances required for mold growth due to high chelating abilities of these agents at low pH values. The second one could

be attributed to disruption of intracellular pH balance of the mold in the presence of these agents.

Table 3. pH values of CZA, PDA and fig-extract agar medium amended with the different concentrations of citric acid, phytic acid, EDTA or Na_2-EDTA. [a]

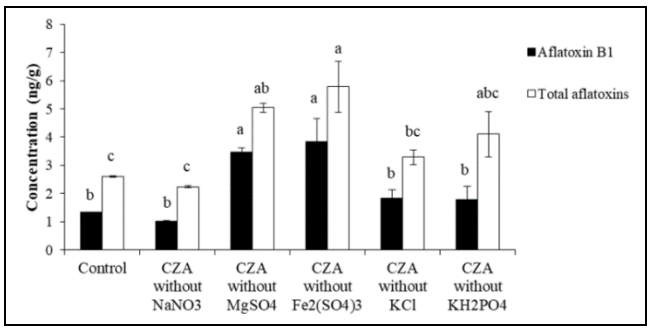
Medium	Chelating agent	Concentration (mM)	pH
CZA	Citric acid	0	7.23±0.01
		0.175	7.12±0.01
		0.875	6.67±0.01
		1.75	6.23±0.01
	Phytic acid	0	7.22±0.01
		0.175	7.08±0.01
		0.438	6.56±0.02
		0.875	5.65±0.01
	EDTA	0	7.21±0.01
		0.175	7.29±0.01
		0.875	6.84±0.01
		1.75	5.69±0.02
PDA	Citric acid	0	5.40±0.02
		0.175	5.39±0.02
		0.875	4.85±0.02
		1.75	4.47±0.01
	Phytic acid	0	5.43±0.01
		0.175	4.92±0.01
		0.438	4.42±0.01
		0.875	3.82±0.01
	EDTA	0	5.39±0.01
		0.175	4.97±0.02
		0.875	4.61±0.02
		1.75	4.29±0.01
Fig-extract agar	Citric acid	0	4.89±0.01
		0.175	4.83±0.01
		0.875	4.77±0.01
		1.75	4.64±0.01
	Phytic acid	0	4.90±0.01
		0.175	4.82±0.02
		0.438	4.72±0.01
		0.875	4.52±0.01
	EDTA	0	4.87±0.01
		0.175	4.62±0.01
		0.875	4.34±0.01
		1.75	4.35±0.01
	Na_2-EDTA	0	4.90±0.01
		1.75	4.79±0.01
		3.50	4.71±0.01
		7.00	4.66±0.01
		8.75	4.61±0.01

[a]: Data represent mean values and standard errors of three replicates.

3.5 The effects of the absence of minerals on aflatoxin production by *A. flavus*

To determine the effects of the absence of minerals on aflatoxin production; modified CZA media prepared by excluding one of the components ($NaNO_3$, $MgSO_4$, $Fe_2(SO_4)_3$, KCl or KH_2PO_4) were inoculated with toxigenic *A. flavus* and incubated for 21 d at 30 °C. The results are shown in Figure 5.

The concentrations of aflatoxin B_1 and total aflatoxins (B_1+B_2+G_1+G_2) determined in the control plates were 1.34±0.00 and 2.61±0.03 ng/g, respectively. The amount of aflatoxins produced in the media did not change significantly in case of excluding $NaNO_3$, KCl or KH_2PO_4 ($p>0.05$), but increased in the absence of $MgSO_4$ or $Fe_2(SO_4)_3$ ($p<0.05$). The concentrations of aflatoxin B_1 and total aflatoxins determined in CZA without $MgSO_4$ were 3.46±0.16 and 5.05±0.16 ng/g, respectively. These values were 3.84±0.81 and 5.75±1.88 ng/g, respectively, for CZA without $Fe_2(SO_4)_3$. In early studies, it was reported that Mg and Fe added to the media caused an increase [23],[58] or a decrease [59]-[61] in the amount of toxin produced by *A. flavus* and *A. parasiticus*.



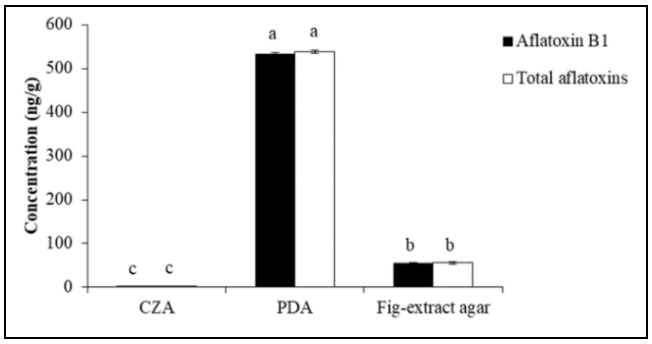
[a]: Data represent mean values and standard errors of three replicates, [ii] For a specific aflatoxin type, values with different letters are significantly different according to Tukey's Multiple Range Test ($p<0.05$).

Figure 5. Aflatoxin production by *A. flavus* in modified CZA media at the end of incubation at 30 °C for 21 d. [a]

We reported that no growth was observed throughout the 6 d incubation period in the medium prepared without $NaNO_3$ and explained it could be due to the lack of the only nitrogen source in the medium. However, when these plates (containing medium prepared without $NaNO_3$) were incubated for a longer period (up to 21 d) to test aflatoxin production, we observed some mycelial growth on the plates. In these plates, we detected 1.03±0.01 ng/g aflatoxin B_1 and 2.24±0.03 ng/g total aflatoxins at the end of 21-d of incubation. This situation may indicate that the mold which could not find any nitrogen source in the medium got stressed and produced aflatoxins. Some factors (drought, lack of nutrients, etc.) that negatively affect the growth of toxigenic *Aspergillus* strains were reported to cause stress on the mold and stimulate toxin production [62],[63]. On the other hand; nitrate, one of the main nitrogen sources used for growth was reported to suppress aflatoxin biosynthesis in toxigenic *A. parasiticus* [64]. Ehrlich and Cotty [65] reported that nitrate could affect aflatoxin biosynthesis via the expression of the key regulatory genes involved in the biosynthesis reactions.

3.6 The effects of different media on aflatoxin production by *A. flavus*

Aflatoxin B_1 and total aflatoxins amounts produced by *A. flavus* in CZA, PDA and fig-extract agar were determined. The results are shown in Figure 6. PDA was the medium in which maximum amounts of aflatoxins were produced ($p<0.05$). The concentrations of aflatoxin B_1 and total aflatoxins determined in this medium were 532.95±3.35 and 538.14±3.58 ng/g, respectively. *A. flavus* was found to produce relatively lower amounts of aflatoxins (53.69±2.56 ng/g aflatoxin B_1 and 55.87±2.58 ng/g total aflatoxins) in fig-extract agar while CZA was the medium in which the minimum toxin production (1.13±0.05 ng/g aflatoxin B_1 and 2.47±0.11 ng/g total aflatoxins) was detected. Likewise, Fakruddin et al. [66] reported that *A. flavus* strains produced limited amount of aflatoxin B_1 (0.03 ng/g) in CZA. Riba et al. [67] detected a maximum amount of only 0.24 ng/g aflatoxin B_1 in Czapek yeast extract agar.



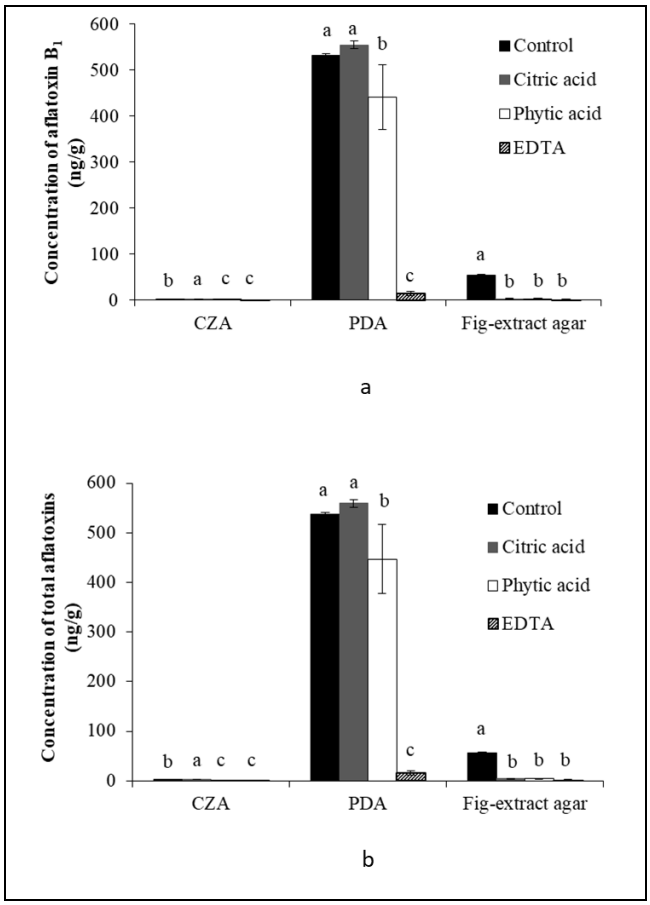
[a]: Data represent mean values and standard errors of three replicates. For a specific aflatoxin type, values with different letters are significantly different according to Tukey's Multiple Range Test (p<0.05).

Figure 6. Aflatoxin production by *A. flavus* in CZA, PDA and fig-extract agar at the end of incubation at 30 °C for 21 d. [a]

The results of the present study suggest that PDA and fig-extract agar are more suitable than CZA for both growth and aflatoxin production of *A. flavus*. This is probably due to the differences in the composition of different media such as carbon and nitrogen sources, vitamin and mineral contents, etc. After conducting experiments on a chemically defined medium, Davis et al. [23] reported that fructose resulted in the fastest growth of *A. flavus* and glucose resulted in the highest amounts of aflatoxins. Furthermore, the authors indicated that the mold grew faster in the presence of amino acids like aspartate, glycine, glutamate, etc.; however, complex nitrogen sources such as yeast extract and peptone, caused higher amounts of aflatoxin production. It is obvious that the compositions of the three media tested in the present study are quite different in terms of carbon and nitrogen sources, vitamin and mineral contents and this is one of the main reasons for observing varied growth rates and amounts of toxins. PDA, made from potato infusion and dextrose, is known as the principal media for fungal cultivation. Fungi can break down starch in potato into soluble sugars which can serve as a source of both carbon and energy. In addition, potato is a complex medium that provides nitrogen, enzymes, vitamins and mineral elements for fungal growth [68]. On the other hand; dried figs, which are the main component of fig-extract agar, were reported to have the best nutrient score among dried fruits [69]. Hence, these two media allowed efficient growth of fungi in the present study.

3.7 The effects of chelating agents on aflatoxin production by *A. flavus*

The most effective growth inhibitory doses of chelating agents (1.75 mM for citric acid and EDTA, 0.875 mM for phytic acid) were tested for controlling aflatoxin production by *A. flavus*. The results obtained for aflatoxin B_1 and total aflatoxins are given in Figure 7a and Figure 7b, respectively. The levels of both aflatoxin B_1 and total aflatoxins were affected with the same trend in response to chelating agent treatments. The effects of chelating agents on aflatoxin production strongly depended on the medium type. For example; in fig-extract agar amended with citric acid, aflatoxin B1 and total aflatoxin amounts were 95.0 and 92.5% less than those in control plates. On the other hand; aflatoxin production amounts did not change in PDA ($p>0.05$) and increased in CZA ($p<0.05$) by addition of citric acid. Aflatoxin B_1 concentrations were 1.68±0.14 and 1.23±0.06 ng/g in CZA with and without citric acid, respectively. The corresponding values were 2.90±0.13 and 2.66±0.19 ng/g for total aflatoxins. Similarly, aflatoxin production was reported to be induced by adding 0.4% citric acid in CZA in an early study [70].



[a]: Data represent mean values and standard errors of three replicates, [ii] For a specific medium, values with different letters are significantly different according to Tukey's Multiple Range Test (p<0.05).

Figure 7. Aflatoxin B_1. (a): and total aflatoxins. (b): Production by *A. flavus* on CZA, PDA and fig-extract agar in the presence of chelating agents at the end of incubation at 30 °C for 21 d. [a]

We indicated that no growth was observed throughout the 6-d incubation period in CZA containing EDTA at 1.75 mM. However, when the incubation period was extended to 21 d to determine aflatoxin production in this medium, we observed a growth of *A. flavus*, even if just limited. EDTA inhibited the production of both aflatoxin B1 and total aflatoxins by 16% in CZA at the end of 21-d incubation. The rates of aflatoxin production inhibition obtained with EDTA and phytic acid in CZA were not significantly different ($p>0.05$).

In conclusion, according to our results, the growth and aflatoxin production of *A. flavus* widely differed according to the media used. Furthermore, the effectiveness of chelating agents in controlling mold growth and toxin production strongly depended on the type of the medium and the agent dose. More than 90% inhibition in aflatoxin production was achieved while mold growth was completely prevented in some cases by the use of chelating agents. All the agents used in this study have been classified as GRAS (generally recognized as safe) by the US Food and Drug Administration (FDA). Moreover; they are all water-soluble so their aqueous solutions can be easily prepared. These solutions can be applied directly to the fruit on the tree or to the soil around the tree. The latter application would be also useful since toxin-producing molds (*A. flavus*, *A.*

parasiticus, etc.) are soil-borne fungi. It is obvious that studies that evaluate the efficacy of these agents against aflatoxin-producing molds in the orchard and investigate the effect of the treatment on fruit quality are strongly needed.

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