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Identification and control of toxin-producing fungus in wheat grain stores using environment friendly factors

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Abstract

Background: The problem of mycotoxins in wheat crops can lead to health issues for humans and animals. It is very important to find safe and efficient methods to prevent the growth of toxin-producing fungi or reduce and remove them from food to prevent their risks to humans, animals, and agricultural crops. The paper focuses on finding environmentally friendly materials to protect wheat grains from toxin-secreting fungi, particularly *Aspergillus niger*, reducing the production of aflatoxin B1.

Methods: Eight fungal genera were isolated from wheat grain samples, and the most frequent fungus was *A. niger*. Plant extracts were used to reduce the growth of *A. niger*, and the culture media was used to test the ability of *A. niger* fungus isolates to produce aflatoxin toxins. The media was also used to sow wheat grains for the purpose of isolating the fungi associated with them, growing the fungi, and purifying it. The culture media was poured into sterile petri dishes with a diameter of 9 cm, with three replicates for each concentration, and the comparison treatment in which an untreated food media was used.

Results: The paper found that *A. niger* was the frequent fungus among the isolated fungal genera in wheat grain samples. The plant extracts, particularly citrullus extract, showed a clear effect in inhibiting the growth of *A. niger* and reduced the AFB1 toxin that it produces. The paper recommends continuous examination of stored wheat grains to determine the amount of fungal contamination and to ensure good storage conditions before receiving the grains in grain stores.

Conclusion: The paper aimed to detect fungi associated with local and imported wheat grains stored in the stores of Al-Muthanna Governorate and the possibility of reducing them by using some plant extracts.

Introduction

Wheat (*Triticum aestivum* L.) is one of the most significant cereal crops since it is the primary food source, contributing to the food security of the world's population [1, 2]. It is the primary food because it is a low-cost source of energy for the body, and its value as food stems from the fact that it includes high levels of nutritious components like protein. Bread wheat includes 11.3 grams of protein, lipids, carbs, fiber, salts, vitamins B1 and B2, and amino acids, and it is incorporated in a wide range of economically significant items [3]. Wheat is one of the most fundamental foods, and its demand grows with population growth. According to estimates, the global population will require one billion tons of wheat by 2020. Wheat, maize, and rice account for 75 percent of the world's grain output [4]. The wheat production in Iraq for the winter season of 2021 was estimated at 4234 ton, with an estimated decrease of 32.1% compared to the year 2020, whose production was estimated at 6238 ton, as the cultivated area throughout Iraq was estimated to be 9464 hectares during the winter season of 2021 [5]. Grain crops are subject to infection with numerous diseases in the field, transportation, and storage [6]. Transmitted fungi are among the most serious diseases on wheat, causing poor germination, low grain quality, and the generation of mycotoxins [7, 8]. The problem of food contamination and its consequences on humans and their animals as a result of ingesting food contaminated with fungus or their toxins is one of the most serious worldwide issues, especially in the field of nutrition. Aflatoxin B1 is the primary cause of liver cancer, AND an established disease that kills infected animals and has carcinogenic consequences on people [9]. *Aspergillus* is one of the most abundant and widespread poisonous fungi in soil, air, water and seeds, and it has economic, environmental and health risks. Its danger lies in its ability to produce aflatoxins and other toxins [10, 11]. According to the statistics of the Food and Agriculture Organization, more than 25% of the global food is contaminated with mycotoxins and that 4.5 million people in developing countries are exposed to quantities of mycotoxins [12]. Due to the high toxicity and high thermal stability of aflatoxin B1, it was classified by the International Agency for Research on Cancer (IARC) as a class 1 toxin [13, 14], so the legal standards set the amount of aflatoxin in foods allowed for human consumption to be approximately four micrograms/ kg for each of B1, B2, G1, G2 combined, and the permissible limit for aflatoxin B1 alone is about 2 micrograms/ kg [15], and the permissible limit for aflatoxins in Iraq is approximately 5 micrograms/ kg as a maximum according to Iraqi specifications 2058 of 1999 [16].

Mycotoxins are one of the most important problems facing the wheat crop, and because of the massive consumption of this crop in Iraq and its economic importance, many things emerge, the most dangerous of which are the health issues for humans and animals as a result of contamination of wheat with AFB1 toxins. This prompted the need to search for highly efficient methods and technologies that do not affect the environment and health in reducing mycotoxins [17,18]. The search for highly efficient and safe means to prevent the growth of toxin-producing fungi or to reduce and remove them from food is necessary to prevent their risks to humans, animals and agricultural crops [19, 20]. The study aimed to obtain environmentally friendly materials [using citrullus extract and phylex] to protect wheat grains intended for storage from toxin-secreting fungi, in particular *Aspergillus niger*, and to reduce the aflatoxin B1 it produces.

Methods

Prepared media and solutions

Potato Dextrose Agar (PDA)

This media was prepared by dissolving 39 g of the ready-made PDA preparation in a liter of deionized water, and according to the instructions of the producing company, sterilization took place in the autoclave for 20 minutes at a temperature of 121 °C and a pressure of 1.5 kg/ cm^2, after the end of the sterilization period, the media was cooled to 45 °C, then the antibiotic (Tetracycline) 50 mg/ L was added to it to prevent the growth of bacteria, and the media was distributed in Petri dishes, each with a diameter of 9 cm. This media was used to sow wheat grains for the purpose of isolating the fungi associated with them, growing the fungi, and purifying it.

Potato media and liquid dextrose

This media was prepared by dissolving 39 gm of liquid potato preparation in a liter of deionized water, and according to the instructions of the producing company, sterilization took place in the autoclave for 20 minutes at a temperature of 121 °C and a pressure of 1.5 kg/ cm^2. After the end of the sterilization period, the media was cooled to 45 °C, then the antibiotic tetracycline 50 mg/ L was added to it to prevent the growth of bacteria, and the media was distributed in glass flasks with a capacity of 250 ml and 100 ml of the media. This media was used to test the ability of *A. niger* fungus isolates to produce Aflatoxin.

Preparation of plant extracts

The plant extract of citrullus was prepared by bringing the fruits of citrullus to the plant pathology laboratory at the College of Agriculture- Al-Muthanna University,

where the fruits were sterilized and placed in a heat-resistant glass beaker containing distilled water in a volume of 1000 ml. It was then subjected to heating until the water reached a boiling point. The fruits were dried, and the pulp was extracted and put in a blender for every 100 gm of the pulp of hibiscus, to which 100 ml of distilled water was added to prepare the standard extract of the hibiscus. As for the phylex extract, it was obtained in the form of a commercial liquid formulation consisting of a group of organic acids, namely formic, lactic, propionic, sorbic and citric. The plant extracts were kept in dark, tightly sealed in bottles and stored in the refrigerator at a temperature of 10 °C until use.

Samples collection

Samples of wheat grains were collected from the Samawah silo, with a weight of 3 kg/ sample, and for four varieties of wheat (a local variety, an Australian variety, a Canadian variety, and a Romanian variety). The random sampling method was adopted by three replications for each variety. It was kept at laboratory temperature until the tests were conducted.

Isolation and identification of fungus discovered on wheat grains

400 wheat grains have been collected from each sample, and divided into two parts, one was not sterilized, while the other was superficially sterilized by soaking them in a 1% sodium hypochlorite solution. After shaking gently for two minutes, the grains were washed three times with sterile deionized water and dried with sterile filter sheets. Five grains were transferred to each petri plate with PDA culture material using forceps that were sterilized under sterile circumstances. The plates were kept in the incubator for 7 days at a temperature of 25 ± 2. After the incubation period, the fungus was diagnosed based on phenotypic characteristics according to the recognized taxonomic keys [21, 22], The proportion of frequency and occurrence was recorded to identify the most common and visible fungus according to the two equations [22]: Occurrence = number of fungal isolates / total number of fungal isolates x 100

Frequency % = number of samples containing gender / total number of samples x 100

Purification of *A. niger* isolate

After identifying the fungus associated with wheat grains visually and microscopically and computing the proportion of frequency and appearance, the isolates of A. niger were the most common and frequent among the isolated and diagnosed fungi. These isolates were purified by transferring the tip of the fungal thread by means of a sterile isolation needle, and from all dishes and samples in which the fungus appeared to dishes containing media PDA, incubated at a temperature of 25±2 for 7 days and kept until subsequent tests are carried out on it [21, 22].

The effect of plant extracts on fungal growth of the pathogenic fungus *Aspergillus niger* in PDA media

The PDA culture media prepared in step (2-1-1) was used and distributed in glass flasks (volume 250 ml) with 100 ml of the nutritional media and sterilized in the Autoclave at a temperature of 121°C and at a pressure of 1.5 kg/ cm^2 for 20 minutes. Before solidification, known quantities of plant extract of citrullus and phylex were added to these flasks containing the culture media, at concentrations (0.0, 1.5, 2.5, 3.5) ml for each extract. The flasks were well shaken to ensure homogeneity of the extract with the culture media. The culture media was poured into sterile Petri dishes with a diameter of 9 cm, with three replicates for each concentration, and the comparison treatment in which an untreated food media was used. After the media hardened in the dishes, the tip of the hyphae was transferred to the fungus taken from the dishes containing a colony of the pathogenic fungus *A. niger*, at the age of five days. All plates were incubated in the incubator at a temperature of 25±2, and growth was measured after 48 hours and after 72 hours of the incubation period, and the growth rate was noted.

Effect of citrullus and phylex extracts on Aflatoxin production of *A. niger*

Potato liquid media prepared in step (2-1-2) was used and distributed in glass vials [volume 250 ml] with 100 ml of liquid media and sterilized in autoclave at a temperature of 121°C and at a pressure of 1.5 kg / cm^2 for 20 minutes. After lowering the temperature, media and known quantities of citrullus and phylex were added to these beakers containing the culture media at concentrations (0.0, 1.5, 2.5, 3.5) ml for each extract, and the comparison treatment in which an untreated food media was used. The fungus disc diameter measuring 0.5 cm in diameter was taken from the pathogenic fungus *A. niger*. All dishes were incubated in the incubator at a temperature of 25 ±2 for a period of 21 days and sent to the laboratories of the Soil and Water Research Department at the Ministry of Science and Technology for the purpose of quantitative assessment of toxins using HPLC and standard poison.

Quantitative determination of aflatoxin B1 in potato liquid sucrose media (PBD) of the fungus *A. niger* isolate by High Performance Liquid Chromatography (HPLC)

According to the method of [23], the process of extracting the toxin from the liquid media of the isolate

A. niger fungus was carried out according to the following steps:

1- The media was filtered with filter paper and the same volume of chloroform was added to the filtrate, and the mixture was placed on a shaker for 1-2 hours.
2- Put the mixture in a separation funnel, shaken gently, and placed the funnel on an iron stand until the separation of the bottom layer contained chloroform.
3- Collected the filtrate and passed it on 1 Whatman filter paper containing 40 gm of anhydrous sodium sulfate per 100 ml. Collected the resulting filtrate and took 25 ml of its volume to measure the concentration of AFB1 by HPLC technology.
4- The aflatoxin B1 toxin was quantitatively estimated in the filtrate after comparison with the standard toxin injected into the device, using the HPLC model [SYKAMN], according to the method of [24] and according to the following equation:

AFB1 concentration = [Standard concentration X Sample area/Standard area] X Dilution/Sample weight

Results

The percentage of occurrence and frequency of fungi isolated from imported and local wheat grains stored in the stores of Al-Muthanna Governorate

The results in Table (1) showed the isolation of eight fungal genera from imported and local wheat grain samples and for different varieties of wheat, namely (the local variety, the Australian variety, the Canadian variety, and the Romanian variety). *A. niger* percentage of occurrence and frequency were 33.33 and 44.45% for the local variety, 33.33 and 33.34% for the Australian variety, 33.33 and 28.58% for the Canadian variety and 66.66 and 50% for the Romanian variety, respectively, followed by *A. parasiticus* with an incidence and frequency of 50, and 44.44% for the Australian variety, 50 and 57.14% for the Canadian variety, and 33.33 and 50% for the Romanian variety, respectively, whereas, *A. parasiticus* did not record any appearance or frequency in the local variety of wheat, while other genera were present in varying proportions among the stored wheat varieties. The above results indicated that the fungus A. *niger* appeared in high proportions in all local and imported varieties.

Effect of different concentrations of citrullus and phylex extracts on the inhibition of the growth of the fungus *A. niger* on the culture media (P.S.A.) After 48 hours of growth

The results of Table (2) showed that there is a clear effect of the citrullus extract in inhibiting the growth of the fungus *A. niger*, as it gave an inhibition rate of 64.3% compared to the phylex extract, which gave an inhibition rate of 37.5%, while the concentration of 3.5% for both extracts had a clear effect in inhibiting the growth of the fungus A. niger, as the inhibition rate was 100%, compared to concentrations 1.5 and 2.5% which gave an inhibition rate of 48.2 and 4.55%, respectively. While the bilateral interaction between the type of extract and the concentrations gave different inhibition rates, the best of which was the interaction between the concentration of 3.5% and the extracts of citrullus and phylex, as the inhibition rate reached 100% for both extracts, compared to the lowest inhibition rate, which was 25% at a concentration of 1.5 and 2.5% with phylex extract.

Effect of different concentrations of citrullus and phylex extracts on the inhibition of the growth of the fungus *A. niger* on the culture media P.S.A. After 72 hours of growth

The results of Table (3) showed that there is a clear effect of the citrullus extract in inhibiting the growth of the fungus *A. niger*, as it gave an inhibition rate of 65.0% compared to the phylex extract, which gave an inhibition rate of 53.6%, while the concentration of 3.5% for both extracts had a clear effect in inhibiting the growth of the fungus *A. niger*, as the inhibition rate was 100% compared to the concentration 1.5%, which gave the lowest inhibition rate of 63.6%. While the binary interaction between the type of extract and the concentrations were giving different inhibition rates, the best of which was the interaction between the concentration of 3.5% and the extracts of citrullus and phylex. It gave the percentage of inhibition as 100% for both extracts, compared to the lowest percentage of inhibition which amounted to 57.1% when the concentration of 1.5 and 2.5% overlapped with the phylex extract.

The effect of citrullus and phylex on the induction of the fungus *A. niger* to the production of Aflatoxin B1 toxins

The results of Table 4: showed that there was a clear effect of the citrullus extract at a concentration of 3.5 % in reducing the AFB1 toxin produced from the fungus *A. niger*, as it gave a concentration of 2.25 ppb compared to the phylex extract, which gave a reduction concentration of 6.0 ppb , compared to the control treatment of 13.9 ppb.

No.	Isolated fungus	Stored wheat varieties							
		Local variety		Australian variety		Canadian variety		Romanian variety	
		%to occurrence	%to frequency	%to occurrence	%to frequency	%to occurrence	%to frequency	%to occurrence	%to frequency
1	*Aspergillus niger*	33.33	44.45	33.33	33.34	33.33	28.58	66.66	50
2	*Aspergillus parasiticus*	0.00	0.00	50	44.44	50	57.14	33.33	50
3	*Penicillium* spp.	16.66	11.11	0.00	0.00	0.00	0.00	0.00	0.00
4	*Alternaria* spp.	16.66	11.11	0.00	0.00	0.00	0.00	0.00	0.00
5	*Fusarium* spp.	0.00	0.00	0.00	0.00	16.66	14.11	0.00	0.00
6	*Trichoderma* spp.	0.00	0.00	16.66	11.11	0.00	0.00	0.00	0.00
7	*Rhizopus* spp.	50	33.33	0.00	0.00	0.00	0.00	0.00	0.00
8	*Aspergillus flavus*	0.00	0.00	33.33	16.66	0.00	0.00	0.00	0.00

Table 1: The percentage of occurrence and frequency of fungi isolated from imported and local wheat grains stored in the stores of Al-Muthanna Governorate.

Extracts type	Extracts concentrations				Means of extracts type
	% 0	% 1.5	% 2.5	% 3.5	
citrullus extracts	0.0	71.4	85.7	100.0	64.3
phylex extracts	0.0	25.0	25.0	100.0	37.5
Means of extracts concentrations	0.0	48.2	55.4	100.0	
L.S.D[0.01[	Extracts type 15.78 = Extracts concentrations 22.31 = Interaction31.55 =				

Table 2: The effect of different concentrations of citrullus and phylex extracts on the inhibition of the growth of the fungus *A. niger* on the culture media P.S.A. After 48 hours of growth.

Extracts type	Extracts concentrations				Means of extracts type
	% 0	% 1.5	% 2.5	% 3.5	
citrullus extracts	0.0	70.0	90.0	100.0	65.0
phylex extracts	0.0	57.1	57.1	100.0	53.6
Means of extracts concentrations	0.0	63.6	73.6	100.0	
L.S.D[0.01[	Extracts type 6.51 = Extracts concentrations 9.21 = Interaction13.02 =				

Table 3: The effect of different concentrations of citrullus and phylex extracts on the inhibition of the growth of the fungus *Aspergillus niger* on the culture media P.S.A. After 72 hours of growth.

Sample number	Sample name	toxin concentration AFB1 (ppb)
1	*A. niger* fungus + liquid media only	13.9
2	*A. niger* fungus + Liquid media treated with citrullus extract, 3.5% concentration	2.5
3	*A. niger.* fungus + Liquid media treated with phylex extract, 3.5% concentration	6.0

Table 4: The effect of citrullus and phylex on the induction of the fungus *A. niger* to the production of Aflatoxin B1 toxins.

Discussion

Observed through a study to detect fungi associated with local and imported wheat grains stored in the stores of Al-Muthanna Governorate under poor storage conditions, appearance of many fungal species contaminating wheat grains, including fungi *A. niger* was observed in high percentages in all local and imported varieties of wheat grains and is consistent with the findings of this cited study [25].

This study showed that there is a clear effect of the citrullus extract in inhibiting the growth of the fungus *A. niger* after 48 hours and 72 hours from the growth of fungi on the nutrition medium. The reason for this may be attributed to the role of citrullus extract in inhibiting the growth of *A. niger.* because it contains many chemical and organic compounds that inhibit the innate growth of fungi, such as alkalis, organic acids, turbines, and others [26, 27]. In addition, the study has given a clear effect of the citrullus extract at a concentration of 3.5 % in reducing the AFB1 toxin produced from the fungus *A. niger* and this indicates the role of inhibitory alkaloids and resins, as well as the role of organic acids in reducing toxins produced by the fungus *Aspergillus* [27].

The paper concludes that plant extracts, particularly citrullus extract, can inhibit the growth of *A. niger* and reduce the production of AFB1 toxin in stored wheat grains. The study recommends periodic examination of stored wheat grains to determine fungal contamination and the need for good storage conditions. The use of plant extracts could be a promising alternative to synthetic fungicides for controlling fungal growth in stored wheat grains. Overall, the study provides valuable insights into the detection and reduction of fungi associated with stored and toxin- producing wheat grains, which could have important implications for food safety and security.

Author Contributions

Ali Faraj Jubair: study design and monitoring of research, Zina Abdulhussein Jawd Abdulhussein: Refinement of study design and supervision and Manuscript scanning, Saad Manee Enad Al-Jabry: Technical support and Data evaluation.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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