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Environmental Causes of Contamination of Warehouse Grains with the *Aspergillus Spp* and the Possibility of Reducing it Biologically

Sarah Alwan Malik Alyasiri
sarah.alwan@uokerbala.edu.iq

Rasha Abd Ulridha Jawad
rasha.jawad@uokerbala.edu.iq

Sabreen abdulhassan
Sabreen.abdul@uokerbala.edu.iq

Doaa Faik Ali Alasady
doaa.f@uokerbala.edu.iq

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ABSTRACT

The study aimed to uncover the most important reasons that lead to contamination of warehouse grains with the Aspergillus spp. Temperature and humidity were the main factors for providing suitable growth conditions for the fungus, which leads to its rapid spread and damage to grains. The study also biologically treated the spread of the fungus using Bacillus bacteria by inhibiting the growth of the fungus and preventing its growth and spread.

Introduction

Biocontrol has emerged as a prominent study focus in recent decades, captivating the attention of scholars as a scientifically sound and ethical approach to managing various plant pests and diseases. This is especially significant given the risks associated with the use of conventional pesticides. (1) The utilization of natural microorganisms is referred to as bio resistance. or pivots in genes or gene products to lessen pest effect and protect crops (2). Bio control goods are those that include various microbial cells. They are commercial preparations that contain live creatures or cells generated from microbes (17). Traditional chemical and physical control methods are uneconomical and environmentally incapable of satisfying future needs (30). Contemporary biological control technologies promote the preservation of main food crops for sustainable global diets. The utilisation of microorganisms in managing various plant pathogens primarily relies on their antagonistic characteristics. The development and application of these microorganisms in the field of biological control are expanding, driven by growing apprehension regarding the use of chemicals and their effectiveness in managing certain pathogens that are resistant to conventional methods. Numerous research have explored the potential use of

microorganisms to control fungal infections in important crops stored under harsh circumstances, including wheat, corn, rice, and other grains. A research discovered that the antibiotic itrin A, which is a lipoprotein obtained from Bacillus subtilis, effectively inhibited the development of *Aspergillus parasiticus* and completely halted the production of aflatoxin at a concentration of 50 ppm (26). Another investigation found that a Bacillus subtilis vaccination biologic was very successful at protecting maize seed against the damaging effects of the fungus *Aspergillus flavus* and *Aspergillus niger* during storage (5). (2) A further investigation showed that treating maize and wheat seeds with Bacillus subtilis with calcium bicarbonate at a dosage of 1 g/kg effectively shielded the examined grains against *Aspergillus flavus* and *Aspergillus parasiticas* infections. The investigation further validated the well-being and physical condition of the animals. Alternatively, people may use it for the sake of food preservation.

General *Bacillus Subtilis* Taxonomy

It's one of the most prevalent Bacillus species, sometimes known as *Bacillus* haliscens or Bacillus phlei. This bacterium's cells are Gram-positive in the aerobic Gram test and are required, albeit occasionally facultative, for anaerobic sporulation. It can survive adverse circumstances such as heat and drought, and it has a sticky feel. It is found in water, soil, and ruminant cavities, and it is an enzyme producer in people. It grows at mesophilic temperatures, with an ideal temperature of 30-25 C for growth (10Specify the genus. Gram-positive Bacillus is common among soil microbes. Its species are heterotrophic, aerobic, moderately heated, endosporic, heat-resistant, and have flagella. Wavy (16).They are extracted from the soil and placed into the feeding medium after being pasteurized. The typical colony look is white to viscous (white and dry or stale), sticky and glossy in texture, rectangular in form, typically in chains or pairs, and microscopically punctate due to plaques inside them. Specific chemicals (such as phytohormones and plant growth regulators) are affected these bacteria release chemicals that directly or indirectly boost plant development, improve soil composition, biologically treat polluted soil, sequester toxic species, and increase their response to disease resistance (28). The economic significance of enzymes is evident in their diverse metabolic, hydrolytic, oxidative, and reductive capabilities. However, the amounts of these enzymes may vary significantly not just across different species but even within strains of the same species. The enzymes amylase, lipase, protease, and cellulose play a crucial role in several industries such as food, pharmaceuticals, textiles, leather, and others. This species' enzymes account for over half of all enzymes available on the worldwide market, with B. subtilis being utilized to ferment soybeans in Japan (21).

Assign this genus (27) to a category based on the provided table (1).

Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Bacillus*

Aspergillus spp.

Aspergillus is a ubiquitous genus whose species are most common and widespread in soil, water, air and seeds, and have important economic, ecological and medicinal values. Although *A. flavus* was described as a plant pathogen in 1920, the fungus gained the most scientific interest after the emergence of turkey disease X in the UK in the mid-1960s, marking the beginning of the field of mycotoxin (15)(23)(9)(12). The importance of this fungus lies in its ability to produce the most potent carcinogenic secondary metabolite, aflatoxin, from which it actually takes its name. In fact, aflatoxin contaminates several crops pre- and/or post-harvest, particularly oilseeds,

including corn, peanuts, cottonseed, and tree nuts, as well as other crops such as cereals, soybeans, figs, almonds, and worldwide of food and feed according to the United Nations' Food and Agriculture Organization (FAO), the direct losses are as high as US$225 million per year, while the indirect effects are in the form of impaired human and animal health (18)(14)(25). *A. flavus* exhibits pathogenicity towards humans, being a primary contributor to conditions such as keratitis, otitis media, pulmonary and systemic infections in individuals with weakened immune systems, as well as cutaneous aspergillosis and aspergillosis in other animals (15)(20). In addition, aflatoxins, especially aflatoxin B1, are potentially lethal at doses of 0.3 mg/kg body weight and are known to cause liver, stomach, and colon cancers (31)(11). As an animal toxin-producing compound, aflatoxin can produce chronic animal toxicity manifested as a reaction with T cells, reducing vitamin K activity and macrophage phagocytic activity, resulting in immunosuppression, reduced feed conversion efficiency, and growth retardation, thereby reducing body weight gain Aflatoxin biosynthesis is controlled by 25 gene clusters, 23 of which are structural genes and 2 regulatory genes, the most important being aflR, which is responsible for most, if not all, transcriptional activation of the structural biosynthetic pathway, and aflS (aflJ), which are thought to be transcriptional coactivators and/or aflR enhancers. (32)(33)

Materials and Procedures

Isolating and identifying *Bacillus subtilis*

Two localities in the Karbala Governorate were chosen, and soil samples of a size of 250 gram were gathered from each location for each of the designated sites at two distinct depths (5 cm and 10 cm). During 10 minutes, 80 ° C of heat must be applied to destroy vegetative cells. Afterwards, 1 g of each soil sample was obtained, and it was used to create a series of dilutions. 10-1-10-12. By using the intended method including a glass diffuser, a volume of 1 ml from the ultimate dilution was extracted and evenly spread among three Petri plates, each containing 20 ml of nutritional agar that had undergone sterilization for 15 minutes at a temperature of 121 °C and a pressure of 1 atmosphere. The dispersion was achieved by the use of a planned approach with a glass diffuser. Subsequently, the occlusion was placed in an incubator and allowed to incubate for 24 hours at a temperature ranging from to - +37 °C. The following variables were analyzed for each soil sample and location after completing the method.

Bacillus bacteria's microscopic characteristics are being studied

Colonies showing *Bacillus* bacterial characteristics were used to select a sample. A smear was prepared using the culture medium, spread on a sterile glass slide, fixed, colored with gram dye, and examined for bacterial cell shape and spore location. The red dye may be used to identify bacteria that are Gram-positive or Gram-negative (6).

Diagnosis of *Bacillus* isolate

Using the Vitek device, the isolate was identified in the lab after sorting the *Bacillus* genus bacterial samples and picking the most effective one.

Aspergillus isolates

Pre-screened and genetically documented samples were collected from local grains and nuts in Karbala Governorate.

Anti-bacterial activity tests

A/blending method

Prepare media (PDA), coconut medium, and nutrient agar) and dispense into three 500 ml capacity flasks at a rate of 250 ml per beaker. The beakers were sterilized in an autoclave at 121 °C and 1 o'clock pressure for 15 min, then cooled slightly before placing in the first beaker. The inoculum volume of *Bacillus subtilis* was 5.0 ml, the second beaker was 1 mm, and the third

beaker was not compared, and then the contents of each beaker were poured into 12 petri dishes. The bacteria-containing block was incubated at 30 ± 2 °C for 24 hours (19). Inoculate 12 plates with aflatoxin-producing *A. flavus* B1 containing *Bacillus subtilis* (nine plates) and three other bacteria-free plates by inoculating a 5 mm diameter disc from one-week-old growth Place the top of the colony in the center of each plate for comparison. 30°C for 7 days.

Then the amount of inhibition of radioactive growth was calculated for each of the studied isolates; By taking the average of two perpendicular diagonals and applying the Abbott equation mentioned in the book on Pesticides (29).

The inhibition ratio was calculated.

$$\text{Inhibition percentage} = \frac{R1-R2}{R1} \times 100$$

R1 = maximum radial growth of a colony of mushrooms grown on plates that do not contain bacteria (control treatment).

R2 = maximum radial growth of a mushroom colony growing on plates containing bacteria.

B/planning method

Prepare the medium for 15 min at a temperature of 121 °C and a pressure of 1 Jo (PDA) at a rate of 250 ml per beaker, the beakers are sterilized in the autoclave), then cool slightly, then the dishes are seeded 24 h later in Bacteria were grown in NB medium and then incubated for 24 hours. I then inoculate with the fungal slices to be examined. A disc (5) mm for each plate is placed in the center of each plate. All dishes were then incubated at 25°C for one week. Then, the inhibition rate was calculated according to the method of(13).

Result and Discus

Isolation and identification of *Bacillus*

Three regions of the Karbala Governorate yielded five Bacillus isolates. Diagnoses for the isolates were made using:

The isolates' phenotypic traits on the culture medium

On the medium of the solid nutritional broth, colonies of bacteria isolates emerged as rounded, white, opaque colonies inclined to a light brown color as the colony aged. The adverbs correspond to what (4) suggested.

Bacterial microscopic characteristics

The bacterial isolates, which were immobilized on glass slides and subjected to Gram staining under a microscope, exhibited colonies consisting of short to medium-length rod-shaped cells. These cells displayed variations in thickness, with some being swollen and others relatively thin. Additionally, the cells exhibited equal ends and tested positive for the presence of Gram stain-forming spores. The wallet is absent, and some spore sites were partially located at the end (semi-terminal) and in the middle (central) (sub-terminal). These characteristics correspond to the morphologies of Bacillus cells and the level of regularity they display, as indicated in (4).

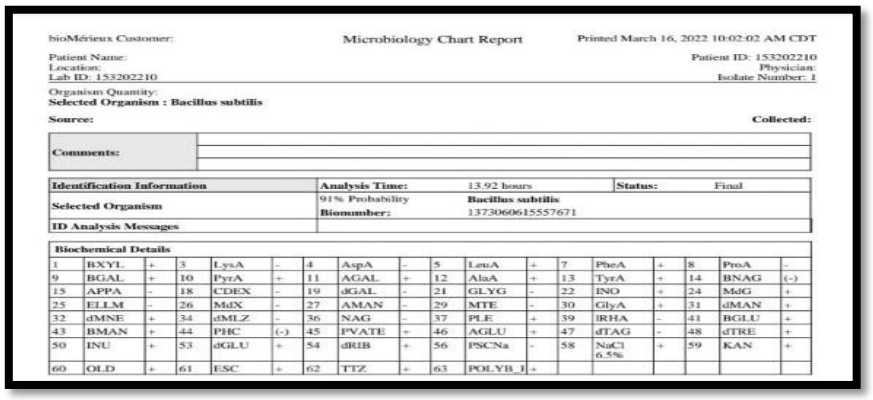
***B. subtilus* phenotypic traits**

Colonies grown by microorganisms on nutritional agar medium N.B. were rather large and had a circular shape, according to laboratory tests. The samples also exhibited a smooth, curved edge that rapidly transitioned into a lobed perimeter as the development advanced. Ultimately, their color spanned from white to light brown and gradually transitioned to a deep brown hue as the growth matured. The colony exhibits a range of diameters, spanning from 1.5 mm to 3.5 mm. This is congruent with what (22) and said (4).

Microscopic description of *B. subtilis*

Upon microscopic examination, the Gram-stained Bacillus colonies were found to be a short gram-positive bacillus that produces sporophytes. During a 24-hour incubation period, these spores are obviously developed and are located in the centre of the bacteria's vegetative cells. The majority of the bacterial cells die after 48 hours of development, releasing the spores. The characteristics match what (4) brought, thus *B. subtilus* bacteria are isolated using biochemical assays.

Figure 1 shows the results of biochemical tests that were conducted on Bacillus subtilus subtype bacterium. when the viteks device was used for the examination



bioMérieux Customer: Microbiology Chart Report Printed March 16, 2022 10:02:02 AM CDT

Patient Name: Patient ID: 153202210
Location: Physician:
Lab ID: 153202210 Isolate Number: 1

Organism Quantity:
Selected Organism : Bacillus subtilis

Source: Collected:

Comments:	

Identification Information	Analysis Time:	13.92 hours	Status:	Final
Selected Organism	91% Probability Bionumber:	Bacillus subtilis 1373060615557671		
ID Analysis Messages				

Biochemical Details

1	BXYL	+	3	LysA	-	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	+	10	PyrA	+	11	AGAL	+	12	AlaA	+	13	TyrA	+	14	BNAG	(-)
15	APPA	-	18	CDEX	-	19	dGAL	-	21	GLYG	-	22	INO	+	24	MdG	+
25	ELLM	-	26	MdX	-	27	AMAN	-	29	MTE	-	30	GlyA	+	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	-	37	PLE	+	39	IRHA	-	41	BGLU	+
43	BMAN	+	44	PHC	(-)	45	PVATE	+	46	AGLU	+	47	dTAG	-	48	dTRE	+
50	INU	+	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	+	61	ESC	+	62	TTZ	+	63	POLYB_I	+						

Figure (1) biochemical tests in viteks test

Diagnostics of *Aspergillus flavu*

Isolation and diagnosis of fungi associated with some types of local grains

A quantity of each sample (nuts and grains) was surface sterilized individually with sodium hypochlorate at a concentration of 2% for two minutes, then washed with sterile distilled water, then transferred directly to container plates on blotting paper for drying, then planted in 9 cm diameter dishes containing sterile (PDA) medium. The dish was cooled using a cooling kit to a temperature of 121°C and a pressure of 1 atmosphere. Then, four seeds were placed along the border of the dish, while a fifth seed was placed in the middle. The plates were cultured at a temperature range of 25-37°C for a duration of 7 days., after the end of the incubation period, the fungal isolates were purified by transferring a 5 mm diameter disk from each colony and planting it in another dish containing the same culture medium. The process was repeated several times to obtain pure fungal isolates The fungi were phenotypically diagnosed according to their phenotypic characteristics based on the phenotypic taxonomic keys (Alis and Pitt, 2009), (Finall and Rapper, 1965), after which the occurrence rate and frequency of each fungus were calculated according to the following equations

$$\text{Appearance rate \%} = \frac{number\ of\ samples\ in\ which\ the\ fungus\ appeared}{R1 number\ od\ total\ sample} \times 100$$

$$\text{Frequency \%} = \frac{number\ of\ fungal\ isolates}{total\ number\ of\ isolates} \times 100$$

Mixing and planning method

The results of these two tests demonstrated the ability of *B. subtilis* to completely inhibit (100%) the growth of both tested fungi. This may be due to the production of many antibiotics by *Bacillus subtilis*, such as acilomycin D, which is an essential antifungal agent for controlling the growth of the *A. flavus* fungus. *Aspergillus* and controls the production of aflatoxins (24) and iturin, a fatty protein that when used at 50 ppm has the ability to inhibit most fungi, as well as degrade fungal cells The secretory wall of enzymes, such as chitin-degrading enzymes and

protease protein-destroying enzymes, help to inhibit radial growth of fungi (7), which is in agreement with (3) finding that *B . subtilis* bacteria inhibit the growth of two fungi, *A. niger* and *A. flavus*, on P.D.A. medium because these bacteria have compounds that inhibit the growth of fungi

Figure No. (2) Inhibitory action of *Bacillus* bacteria by mixing and planning methods for *Aspergillus spp*

Figure No. (3) Inhibitory action of *Bacillus* bacteria by mixing and planning methods for *Aspergillus spp*

The results showed the possibility of using *Bacillus* bacteria in fogging grain stores to limit the growth of *Aspergillus* spp, as this bacteria possesses a high antioxidant capacity, in addition to the ability of calcium carbonate to reduce moisture content, which reduces the chance of growth and spread of the fungus. Therefore, the study urges the use of bacteria loaded with carbonate in fogging warehouses to reduce pollution because it is not possible to control moisture content and temperatures due to the warehouses' lack of good and controlled storage conditions.

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