Original Article

Isolation And Identification Of Fungi Associated With Walnuts, Almonds, Hazelnuts, Cashews, Pistachios And Peanuts Using Molecular Diagnostics Technique(PCR) In The Local Markets Of Kirkuk Governorate / Iraq.

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Abstract

The aim of the current study is to Isolation and identification of fungi contaminated with walnuts, almonds, hazelnuts, cashews, peanuts and pistachios using molecular diagnostics (PCR). Nuts samples were collected with an amount of 2000 grams of walnuts, almonds, hazelnuts, cashews, pistachios and peanuts from different locations of the local markets in the city of Kirkuk and intended for human consumption for the period. From 15-November-2022 until 20-December-2022.The results of the molecular diagnosis Polymerase Chain Reaction (PCR)of the fungi with a sequence analysis and drawing the genetic tree to compare the isolated and diagnosed fungi with the global isolates associated with the fungal genera Aspergillus, Penicillium, Rhizopus, Paecilomyces of nuts, Aspergillus flavus species With a percentage of 26.3%, the isolated fungi sequence and prevalence rates were as follows: A.flavus, A.niger, P. aurantiogrise, P. oxalicum, A. fumigatus, Rhizopus stolonifer and Paecillomyces variotti at 26.3%, 19.7%, 15.8%, 13.8%, 11.2%, 9.2% and 3.9%, respectively.

Key words: walnuts, almonds, hazelnuts, cashews, pistachios and peanuts.

INTRODUCTION

all kinds of Nuts are healthy and useful foods that are classified globally among the important foods. They contain integrated nutrients from proteins, fibers, beneficial unsaturated fats, minerals and vitamins that make them a healthy meal, in addition to containing large amounts of phytochemicals with antioxidant properties that protect the human body. From cancer and heart disease, nuts are consumed in raw or processed form as a whole food and are also included in medical preparations (1).

Due to the high content of nuts of fats and proteins, they are subject to damage and attack by a number of fungi. Nuts may be contaminated either due to the growth of fungal colonies on them or the production of mycotoxins. Usually, raw materials are more likely to obtain greater levels of pollution compared to the final product intended for direct human consumption. (2),as it is exposed to fungal infections in the field and during transportation and storage in the absence of appropriate methods and conditions for storage, which reduces its quality and safety(3). Fungal contamination may occur in nuts in three different stages: either before harvest when the nuts are ripe and their husks open, which are often attacked by airborne fungi where insects help transport them, or after harvest when the nuts are peeled, washed and sorted Wash water can be a source Pollution and if the nuts are left wet, they will also be highly susceptible to the growth of fungi and mycotoxins, and contamination may occur at the storage stage when the appropriate temperatures and humidity are available for the growth of fungi(4). The diagnosis and classification of fungi based on their phenotypic characteristics gives accurate results (5), but sometimes errors may occur in the diagnosis, especially in largely similar fungal groups (6). DNA markers are now one of the modern methods of choice for Distinguishing closely related organisms (7) According to previous studies, the most common fungi in nuts are Fusarium, Aspergillus, Penicillium, Rhizopus, and Alternaria (8).

MATERIALS AND WORKING METHODS

Sample Collection

Six samples of raw and unsalted nuts were randomly collected, including walnuts, almonds, hazelnuts, cashews, pistachios and peanuts by purchasing five replicates of each type from five different vendors for each geographical area at a rate of 100 g/sample to prepare one compound sample for each type. With 2 kg of local markets for four different geographical areas of Kirkuk governorate, where these types of nuts were selected depending on the availability in the market and the popularity of use starting from 11/15/2021 to 12/20 2022, the samples were kept in double sterilized polyethylene bags, and the place and date of collection were recorded on them and kept in the refrigerator at 3-5 °C until use.

ISOLATION AND PURIFICATION OF FUNGI

Nuts samples were transferred to the Graduate Studies Laboratory and were superficially sterilized with 4% sodium hypochlorite solution for two minutes, then washed with sterile distilled water, dried with filter paper, and then planted in sterile Petri dishes with a diameter of 9 cm and containing aquariums. Potatoes and dextrose After adding 250 mg/l of the antibiotic chloramphenicol to prevent bacterial growth, the seeds were placed in the dish and the process was repeated ten times for each type of nuts., after the end of the incubation period, the isolates of fungi were purified by take a swabbing of the fungi spores and transferring them to a new dish containing potato dextrose akar medium and incubating at a temperature of 25 °C for seven days and the process was repeated several times until obtaining pure isolates.

DIAGNOSIS OF FUNGI ISOLATED FROM NUTS MICROSCOPIC DIAGNOSIS

The light microscope was used to diagnose the types of fungi contaminating the nuts samples, after swabbing the purified isolates of the fungi on a medium of potatodextrose akar on the slides using Lacto Phenol Cotton Blue Stain. The shapes and colors of colonies and the phenotypic and microscopic characteristics of each species were observed and compared with the taxonomic keys of the fungi (9) to reach the isolated fungi species.

PCR MOLECULAR DIAGNOSTICS

Polymerase chain reaction (PCR) was performed to investigate the fungal isolates, the fungus DNA was extracted and the target region (ITS) was amplified using each of the primers, ITS 1 (5'-TCC GTA GGT GAA CCT GCG G- 3') and ITS4(5'-TCC TCC GCT TAT TGA TAT GC-3') This diagnosis was made according to (10)

TOTAL DNA EXTRACTION

Deoxyribonucleic acid was extracted from a single pure and active colony of fungal isolates using a Chelex®100 kit made of Chelex®100 solution from BioRad in the USA, and the extraction was performed according to the manufacturer's instructions.

PREPARATION OF THE REACTION MIXTURE FOR PCR

The reaction mixture was prepared from Polymerase Chain Reaction PCR (25 µl) according to the manufacturer's instructions ,Then the components of the PCR reaction mixture mentioned were placed in special 0.2ml tubes containing the rest of the PCR reaction components. All tubes were transferred to the Vortex Centrifunge Exispin at a speed of 3000 cycle / minute for three minutes and then placed in the PCR Thermo Cycler Condition to carry out the process of DNA amplification according to the ideal conditions for thermal cycles for all genes according to the method of work of the manufacturer as in Table1.

Table1. Optimal thermal conditions for PCR stages for the ITS gene.

Time	Temperature	Repeat cycle	PCR step
33 minute	95 °C	1	**Initial denaturation**
30 minute	95 °C	35	**Denaturation**
1 minute	56 °C	1	**Annealing**
5 minute	72 °C	1	**Extension**
50 minute	72 °C	1	**Final extension**

RESULTS AND DISCUSSION

Microscopic Diagnosis

The forms of the developing and isolated fungal isolates on PDA medium, as well as the forms of Conidiaphore and Conidia and the quality of branches in Conidiaphore as well as colony sizes and colors and the colors of the inverted plate for each of them were found to contain the types of fungi Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus ,Penicillium Oxallicum, Penicillium aurantiogrise, Rhizopus stolinifer and Paecilomyces variotii . And that is after comparing the mentioned traits with what was mentioned in the taxonomic keys (11).

INSULATION RATIO FOR TYPES OF FUNGI

This study showed the isolation of several types of fungi associated with walnuts, almonds, hazelnuts, cashews, peanuts and pistachios, which were collected from local markets in the center of Kirkuk governorate, which are prepared for consumption by humans. The isolation results showed that 152 isolates were isolated. From six samples, which belong to four fungi genera, 57.2% of the fungi for the genus Aspergillus sp., 29.9% of the fungi for the genus Penicillium sp., 9.2% of the fungi for the genus Rhizopus sp. and 3.9% of the fungi for the genus Paecilomyces sp. The fungus A.flavus recorded the largest number of isolates 40 isolates, while the fungus Paecilomyces variotii recorded the minimum number of 10 isolates Table 2 .

Table2. Fungi isolated from several types of nuts

No	Number of isolates	isolated fungi	Isolate%
1	Aspergillus flavus	40	26.3
2	*Aspergillus niger*	30	19.7
3	*Penicillium aurantiogrise*	24	15.8
4	*Penicillium oxallicum*	21	13.8
5	*Rhizopus stolonifera*	14	9.2
6	*Aspergillus fumigatus*	17	11.2
7	*Paecilomyces variotii*	6	3.9
	Total	152	100

The fungi of the genus Aspergillus sp. Of the most common pollutants of nuts, it was shown in Table2. that A.flavus, A.niger, Penicillium sp. The most visible isolates of fungi were in all types of nuts, and the highest percentage of appearance of fungi was in peanuts and by 25% for A.flavus, 23.5% for A.fumigatus, 23.3% for A.niger, 21.4 for R.stolonifer, 20.8% for P. aurantiogrise, 19% for P.oxalicum and the lowest incidence of Paecilomyces variotii was recorded at 16.7% for peanuts and cashews, 14.3% for P.oxalicum in hazelnuts, 12% for A.flavus in walnuts and 5.9% for A.fumigatus in almonds and pistachios.

Table3. Percentage of appearance of fungi isolated from nuts

Sample	***A.flavus***	***A.niger***	***A.fumigatus***	***P.aurantiogrise***	***P.oxalicum***	***R.stolonifer***	***P.variotii***
peanuts	25.0	23.3	23.5	20.8	19.0	21.4	16.7
Cashew	17.5	23.3	17.6	20.8	23.8	35.7	16.7
Almond	12.5	16.7	5.9	0.0	23.8	14.3	33.3
Walnuts	12.0	13.3	47.1	25.0	19.0	0.0	0.0
Hazelnuts	17.5	16.7	0.0	20.8	14.3	28.6	0.0
Pistachio	12.0	6.7	5.9	12.5	0.0	0.0	33.3

The results of the frequency ratio of isolated fungi Table 4. The highest frequency of A.flavus was 38.4% in pistachio, followed by A.fumigatus with 28.6% in walnut, 25% in A.niger and P.oxalicum, and 21.4% in P. aurantiogrise in walnut, while the lowest frequency of isolates was for Paecilomyces variotii in peanuts 2.9%, cashew 3%, almond 10%, pistachio 15.4%, respectively.

Table 4. Frequency percentage of fungi isolated from nuts

Sample	***A.flavus***	***A.niger***	***A.fumigatus***	***P.aurantiogrise***	***P.oxalicum***	***R.stolonifer***	***P.variotii***
Peanuts	29.4	20.6	11.7	14.7	11.7	8.8	2.9
Cashew	12.2	21.2	9.1	15.6	15.6	15.6	3.0
Almond	25.0	25.0	5.0	0.0	25.0	10.0	10.0
Walnuts	21.4	14.3	28.6	21.4	14.3	0.0	0.0
Hazelnuts	29.2	20.8	0.0	20.8	12.5	16.7	0.0
Pistachio	38.4	15.4	7.7	23.1	0.0	0.0	15.4

Our results are in agreement with the findings of (12)that A. niger and A. flavus are the most contaminating fungi for nuts, and with what (13)showed by isolating A.flavus and A. niger from walnuts, cashews and pistachios, and with the results from (14) that the fungi of the genus Aspergillus are one of the most contaminated isolates of nuts, with A.flavus giving the highest incidence in field pistachios 80%, and 60% in sunflower, pistachio nuts, pumpkin seeds and walnuts.

Contamination of food products depends on the nature of the food components that make the food suitable for the growth of fungi, as well as environmental conditions such as humidity, hydrogen and temperature(15).

The results of our study showed that the fungi of both genus Penicillium, Aspergillus. It is one of the most common fungi in different types of nuts due to the ability of this fungus to produce a wide range of enzymes that enable it to grow on different nutrients (16,17), as well as its ability to adapt Large with critical environmental conditions. and its ability to produce a large number of panels that have the ability to withstand unfavorable environmental conditions (18).

MOLECULAR DIAGNOSIS OF FUNGI USING POLYMERASE CHAIN REACTION (PCR) TECHNOLOGY.

The test was carried out using polymerase chain reaction (PCR) technique to confirm the diagnosis of the selected fungal isolates based on the specific primer. Isolates were classified by conventional methods on PDA medium, and molecular diagnosis of fungal isolates was confirmed and confirmed to take advantage of modern diagnostic methods using (ITS1) (5'-TCC GTA GGT GAA CCT GCG G-3'), Internal transcribed spacer 4 (ITS4) (5'-TCC TCC GCT TAT TGA TAT GC-3') in the ITS region as an approved taxonomic and genetic indicator for all fungal species as described by(10) by amplification of specific diagnostic loci in the fungi genome as reported by(19), Figure1. The PCR technique was used in the current study to diagnose fungi isolated from nuts, as indicated by (20) that the traditional methods adopted in the diagnosis of fungi and based on the identification of phenotypic criteria are no longer sufficient due to the overlap of these criteria with Other species in addition to the genetic difference between them. Their growth and phenotypic characteristics do not necessarily imply the difference in genotype, especially between farms and isolates of the same genus and species.

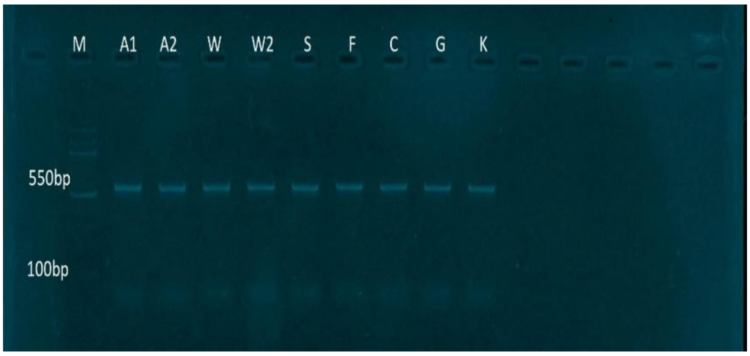

Figure1. Electrophoresis using agarose gel, which shows the results of DNA PCR examination of fungal isolates by amplifying the ITS gene, which includes both the ITS1 and ITS4 primers.

The results of the molecular diagnosis of fungi by PCR technique in our study showed that the tested samples belong to the fungi Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Penicillium oxallicum, Penicillum aurantiogrise, Rhizopus Stolonifer and Paecilomyces variotii.

DNA SEQUENCING ANALYSIS RESULTS

The results of the nucleotide sequence analysis of multiplexed DNA bundles using the National Center for Biotechnology Information (NCBI) program and their comparison with the data available at the National Center for Biotechnology Information NCBI (21).

Sequencing was performed for the selected isolates and entered into the NCBI BLAST program to find out the similarity between them and the results in the global genebank after obtaining the nucleotide sequence of the DNA package of the local isolates and comparing them with the sequences of the same region for the global and local fungal isolates registered in (NCBI BLAST) where Aspergillus flavus showed a concordance of 99.08% with the results in the genbank, Rhizopus Stolonifer showed a concordance with the genbank with a percentage of 97.74%, Penicillium oxalicum gave a match of 96.81%, Aspergillus niger had a match of 96.01%, and paecilomyces variotii had a concordance of 95. 81% and Aspergillus fumigatus 93.66%, while Penicillum aurantiogrise showed a match of 83.70%, indicating that the isolates of this pathogenic fungus are widely spread in the world.

Enter the nucleotide sequence of the DNA bundle in the NCBI BLAST program to find out the similarity between it and the results in the Genebank. The results of the sequence analysis of nitrogenous bases compared to the isolates registered in NCBI proved that all isolates confirm the result of phenotypic and microscopic diagnosis, Table5.

Table5. Global isolates and accession numbers in NCBI compared with BLAST and matching percentage with the isolates under study.

Isolation Number	Molecular Diagnostics	Similarity %Ratio	Accession Number
A1	*Paecilomyces variotii*	95.81%	**KC411910.1**
A2	*Penicillum aurantiogrise*	83.70%	**HG326290.1**
W2	*Penicillium oxallicum*	96.81%	**GU723433.1**
F	*Rhizopus Stolonifer*	97.74%	**MK989600.1**
C	*Aspergillus niger*	96.01%	**MK693451.1**
G	*Aspergillus fumigatus*	93.66%	**MG279206.1**
K	*Aspergillus flavus*	99.08%	**MN533811.1**

GENETIC TREE

A phylogenetic tree based on ITS-1 and ITS-4 region sequences of local fungal isolates was analyzed and compared with sequences of the same region for some global and local isolates of fungi registered in NCBI. Genetic tree analysis was performed in MEGA X (22), Figure2.

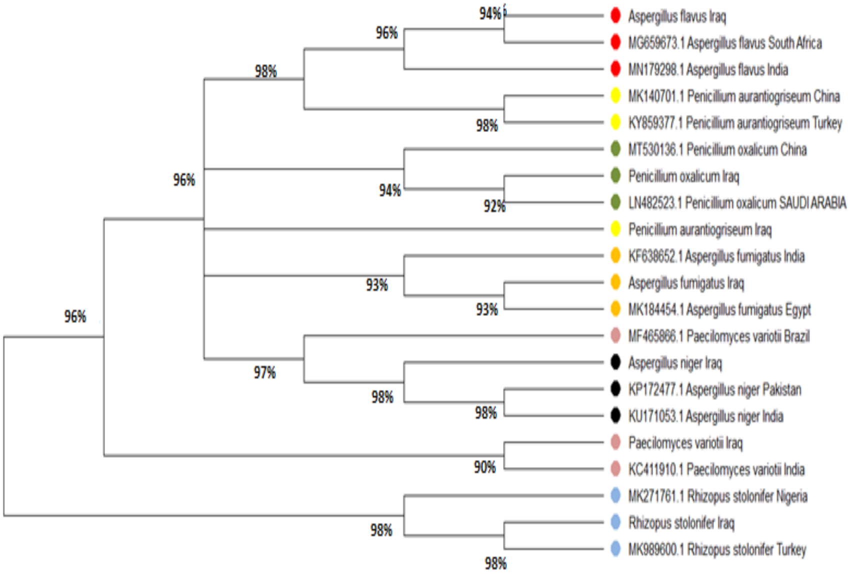

Figure 2. The genetic tree of isolates of fungi that was constructed based on the sequence of their nitrogenous bases.

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