Diagnostic and environmental study of *Aspergillus terreus* isolated from various varieties of apples fruits

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Objectives This study includes the fungi isolated from apple fruits and diagnosis study with ecological factors for Aspergillus terreus.
Methods The samples were collected from apple varieties which included red importer apples, red local apples, golden yellow importer apples, green importer apples. The collected apples had some obvious lesion or spoilage and isolated number of fungi that differences in appearance percent and used two primers SEQ ID NO and ITS for *A. terreus* diagnosis by using PCR technique; in addition the study includes using three factors effective on fungal growth with temperature 15, 20, 25, 30 and 35 C° with pH 4, 6, 8, 10 and 12 and the third factor culture medium PDA, SDA, CZA and MEA.
Results Results revealed the presence of nine types of fungi which includes *P. expansum, Rhizopus solonifer, Alternaria* spp, *A. flavus, A. niger, A. terreus, P. digitatum, P. italicum* and *yeast* isolated from various apple fruits. Number of isolates *P. expansum* more than other fungi while less number was found in *Rhizopus stolonifer*. The *A. terreus* identified by amplicon size of that appear 100 bp for six isolates and it appears that four isolates carrying this gene has size of 600 base pairs. This study showed significant differences ($p < 0.05$) in effect of five temperatures 15, 20, 25, 30 and 35°C in the growth of fungus *A. terreus* and pH levels on growth on three isolates of genus the types of culture media (PDA, SDA, CDA, MEA) significant at $p < 0.05$ affected the growth of *A. terreus* and it had different morphological characteristics.
Conclusion Number of fungi was found in apple and identified *A. terreus* by PCR method and studied different ecology factors on growth fungus. The best degree for the growth of isolates in the sixth day was 30°C. Maximum level of growth of the *A. terreus* was at pH 6 for all isolates in the sixth day and the growth rate for the same day on SDA reached 7.13 cm which was considered the best media for growth.
Keywords *A. terreus*, apple fruits, PCR technics, environment factors

Introduction

The fungi is one of the pathogens that cause damage to the fruits and vegetables after harvest[1] where the plants get injured during the harvest, marketing, packaging and storage. The infection begins during wounds that you get during handling and harvesting.[2] The apples infected by many types of fungi that cause rot in fruit especialy during post-harvest especially by fungi *Pencillium expansum*, *Monilininia fracticola*, *Mucor piriformis*, *Alternaria* ssp., *Colletrichum denatum Botrytis cinerea*, the type of fungi as *P. expansum*, *Botrytis cinerea*, *Alternaria* ssp may it caused a very large economic losses in Italy.[3]. The apples are rich in salts and vitamins and nutrients, where apple medium-sized contain with peel (182 g), according to the US Department of Agriculture fat 0.31%, 0.47%, carbohydrates, proteins 25.13%, fiber 4.4%, sugars 18.91%, and the fruit of apples contains 4.46% kind of vitamins A, B, E and folic acid.[4] Among the benefits of the apples to strengthen the brain, heart, gums its seeds are used to kill the abdomen worm and prevent constipation and indigestion. Apples have nutritional values and it is a compromise natural catalyst for the growth of microorganism, especially fungi, where apples are exposed during the harvest, storage and transport to scratches which facilitates the entry of fungi to the texture of the mature fruit and then putrefaction and obtain significant economic losses causing rotting of fruits due to fungus *A. terreus* during storage and marketing.[5] On the other hand, the fungus events to rot in fruit, especially apples stimulates some strains of the fungus to secrete carcinogens mycotoxins. The best ways that reduce crop yields and food mycotoxin contamination with mycotoxin is by preventing appropriate conditions for fungal growth and production of toxins as well as the need to examine all the food processed for human consumption to make sure they do not contain the mycotoxins which harmful to human health[6, 7]. This study included isolates and diagnosed fungi associated with the fruits of infected apples imported to local markets.Babylon's traditional methods and molecular diagnosis of isolates of fungus *A. terreus* and also the impact of environmental conditions for the fungus are under study.

Materials and Methods

This study was done in the laboratory of mycotoxins and bio-technology in the Department of Biology.

Sample Collection

Samples of apples were collected from different local markets between October and December of 2015 in the province of Babylon. Ten kilograms of the samples were collected for each class of varieties under study places. These included red importer apples, red local apples, golden yellow importer apples, green apple importer apples. The collected apples had some obvious lesion or spoilage. Each apple was placed in a sterile plastic bag in room temperature (25–30 C) for 6 days or until fungal growth was evident all over the sample. The purpose was to investigate the frequency of the presence of fungus in apples.

The types of culture media which was used in this study were (1) Potato Dextrose Agar (PDA), (2) Czapeck's Agar with Sucrose (CDA20S), (3) Sabourauds Agar (SDA) and (4) malt Extract (MEA).

Isolate and Diagnose Fungi Associated with the Apples Under Study

Detection of microorganisms was performed using a moist chamber method. The transfer of apple samples to a laboratory to isolate and diagnose fungi then cut each apple into five small pieces and put in petri- dishes (diameter 9 cm) containing the PDA for each class separately and repeated the process three times (replicates) for each class of the apples then incubated all

dishes under a temperature of 25 ± 2°C for 7 days.[8] Developed fungi were identified by their morphological features according to the previously described methods[6] and all ratios were calculated according to the following equation:

$$\text{The percentage of appearance (\%)} = \frac{\text{the number of samples that have fungus}}{\text{total number of isolates}} \times 100$$

Study of Effects of some Ecological Factors in the Growth of A. terreus

Temperature: A series of temperature were arranged (15, 20, 25, 30 and 35) for the growth of *A. terreus* in sterile petri dish which contains 20 ml PDA and the plate was inoculated in a disc 10 mm from the edge of the colony and incubated dishes for 6 days under 25°C (three replicates). Radial growth (colony diameter) was estimated at 2 days interval till full expansion of the growth on different temperature treatment was reached.[9]

pH: Various pH were arranged (4, 6, 8, 10 and 12) for growth of *A. terreus*. The PDA was divided Pon five flasks glass size 500 ml to 250 ml container, the 4 and 6 by adding drops of hydrochloric HCl acid (5 mm) and a pH of 10 and 12 by adding drops of sodium NaOH solution (40%). Inoculation steps, incubation condition and growth measurement of fungal isolates were conducted as mentioned earlier.[10]

Effect of culture media: To obtain a suitable medium for enhanced growth by *A. terreus* various media were arranged (PDA , SDA, CDA and MEA) in sterile petri dish. Inculcation steps, incubation condition and growth measurement of fungal isolates were conducted as reported above.[11]

DNA extraction and sequence analyses: After 7 days, growth of fungal colonies on PDA was counted and recorded in a colony forming unit per millilitre (cfu/ml). DNA was extracted from isolates using the genomic DNA purification Kit (Promega, Madison, WI, USA). Small subunit ribosomal RNA (mtSSU rRNA) and β-tubulin were then amplified by PCR using primer pairs ITS1/ITS4 primers (ITS1/TTC GTA GGT GAA CCT GCG G) and (ITS4/TCC TCC GCT TAT TGA TAT GC) and (SEQ ID NO: 2F/ACA TGA ACC CTG TTC TGA AAG) and (SEQ ID NO: 2R/CCA AGA GAT CCA TTG TTG AAG) the conditions described[12] here are primers for *A. terreus* diagnosis. For detection of DNA, gel electrophoresis method was used by using Agarose gel (1% concentration). This method consisted of three steps: preparation of agarose gel, preparation of casting agarose and the addition of samples.

PCR amplification condition: This method is used to amplify DNA by using the specific primer. These methods are dependent on the volumes that are found in Master Mix provided from Bioneer (Tables 1, 2).

The time and vol. which is used for electrophoresis methods: To obtain clear band after PCR running , that used different time and volume during electrophoresis.

Results

Table 3 showed that there are many types of fungi in apple varieties, pathogenic fungus *P. expansum* was recorded high in percentage with *P. italicum* appearance in most apple varieties (Table 3). The highest proportion of the emergence of *P. expansum* amounted to 90% in the red apples imported, and the lowest rate of 30% in the appearance of golden yellow apples imported. *A. terreus* was in highest percentage imported. *P. digitatium* was the highest rate imported (20%), but did not appear in green apple imported. *P. digitatium* was the highest rate in green apple imported (60%) and the lowest rate appearance was in the golden yellow apples imported at a rate of 30%. The percentage of the emergence of *Altrenaria* spp. in red apple domestic and golden yellow apple imported as 30% did not appear in green apples either. *A. niger* was the highest proportion of its appearance in the golden yellow apple imported and that 60% did not appear in green apple imported. *A. flavus* was at high percentage in the golden yellow apples imported did not appear in green apple imported.

Table 1. **Programme of PCR technique SEQ ID No. for *A. terreus***

No. steps	Steps	Temperature	Time	No. of cycles
1	Initial denaturation	94°C	5 min	1
2	Denaturation	94°C	30 min	
3	Annealing	50°C	45 min	35
4	Elongation	72°C	1 min	
5	Final extension	72°C	5 min	1

Table 2. **Programme of PCR technique ITS for *A. terreus***

No .steps	steps	Temperature	Time	No. of cycles
1	Initial denaturation	95°C	120 min	1
2	Denaturation	95°C	5 min	
3	Annealing	60°C	45 min	45
4	Elongation	72°C	1 min	
5	Final extension	72°C	5 min	1

Table 3. **Percentages of appearance of fungi isolated from apple varieties**

	The percentage of appearance (%)								
Types of apple fruit	***P. expansum***	***P . italicum***	***P. digitatum***	***A. terrus***	***A. niger***	***A. flavus***	***Alternaria* spp**	***Rhizopus stolonifer***	**Yeast**
Red imported apples	90	40	50	5	30	50	20	10	20
Red local apples	60	80	40	5	20	60	30	20	40
Golden yellow apples imported	30	50	30	20	60	70	30	10	70
Green apple imported	40	70	60	0	0	0	0	0	10

As for yeasts the highest rate in the emergence of golden yellow apples by 70% and the lowest percentage of appearance in the green apple imported by 10%.

Identification of A. terreus based on Molecular Characteristics

The results shown in Fig. 1 are the isolated fungi and gene coding for SEQ ID amplified by PCR technique. Eight isolates of *A.terreus* whose diagnosis by using specialized primers SEQ ID 2F: ACA TGA ACC CTG TTG TGA AAG opposite the primer SEQ ID NO: CCA AGA GAT CCA TTG TTG AAA. The amplicon size of *A. terreus* appear 100 bp for six isolated. As well as the universal pair primer was successful in amplification of ITS region for different isolates of *A.terreus* had been appears that 4 isolates were carrying this gene has size of 600 base pairs in lane (2, 3, 4,5) (Fig. 2).

Effect of some Ecological Factors in the Growth of A. terreus

Temperature

The results of this experiment (Table 4) demonstrated that the growth of *A. terreus* varied according to the level of temperature. This study showed significant differences ($p < 0.05$) with effect to five temperatures 15, 20, 25, 30 and 35°C. The best degree for the growth of isolate At-1 on the sixth day was 30°C, with the growth rate of 9 cm, followed by 35°C growth rate of 6.66 cm. For isolate At-2 the best temperature was 30°C with a growth rate of 7.16 cm in diameter, while isolate At-3 had the best growth on the sixth day at 30°C with growth rate of 8.6 cm. As the results showed in Table 4 the temperature of 25°C all isolates have less growth in the sixth day with 2.83, 3.21 and 3.13 cm, respectively. But at temperatures of 15°C and 20°C caused reduced growth rates in all isolates of *A. terreus*.

pH

The results showed that test three isolates of the *A. terreus* had variation in growth rates through the use of four pH (Table 5) and there were significant differences ($p < 0.05$) in the growth of *A. terreus* on PDA at a temperature of 25 ± 2°C. Maximum level of growth of *A. terreus* was at pH 6 for isolates Af-1 and Af-2 in the sixth day with 6.23 and 7.83 cm, respectively, and radial growth for isolate Af-3 increased at pH 6 (6.23), followed by growth at pH 8, which amounted to 5 cm and 6.23 cm and 4.33 cm for isolates Af-1 and Af-2 and Af-1, respectively. But all the isolates will not growth at pH 4 and 12, except Af-3 which will have little growth at pH 12.

Culture media

The results demonstrated that the type of culture media (PDA, SDA, CDA, MEA) significant ($p < 0.05$) affected the growth of *A. terreus* and its different morphological characteristics was evident that most of the isolates were of colonies of brown colour and characterised isolates at PDA and SDA were light brown while on MEA it was reddish brown colour and CDA was dark brown (Fig. 3). The growth rate in the sixth day of isolate At-1 was 5.75 cm on PDA while the growth rate for the same day on SDA reached 7.13 cm, and 6.23 cm growth on CDA. The results (Table 6) showed the percentage growth on MEA was 6.36 cm, but for the isolate At-2 growth rate at SDA in the same day reached 8.65 cm while it was 7.86 cm on CZA in the same day

Fig. 1 **Gel electrophoresis of amplified PCR product from different *A. terreus* isolate, by using SQE primer (in Vol. = 100, agarose = 0.4%).**

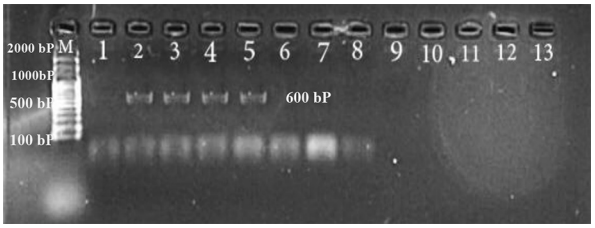

Fig. 2 **Gel electrophoresis of amplified PCR product from different *A. terreus isolates* by using ITS primer (in Vol. = 100, agarose = 1%)**

Table 4. **Effect of different temperature on the growth of *A. terreus* isolates on PDA after 6 days of incubation at pH 7**

Isolates	Days	Temperatures				
		15°C	20°C	25°C	30°C	35°C
		Diameters colonies (cm)				
At-1	2	0	0	2.5	3.26	2.9
	4	0	0	2.5	6.66	4.55
	6	1.51	0	2.83	8.33	6.66
At-2	2	0	0	2.16	2.9	3
	4	0	1.33	2.8	5.5	3.66
	6	0	1.5	3.21	7.16	4.33
At-3	2	0	0	1.66	3.33	3
	4	0	0.9	2.58	6	4.66
	6	0	1.3	3.13	8.6	6.5

LSD value for temperature = 0.569.
LSD value for incubation days= 0.445.
LSD value for overlap = 1.707.

Table 5. **Effect of different pH on the growth of *A. terreus* isolates on PDA after 6 days of incubation at a temperature of 25 ± 2°C**

Isolates	Days	Hydrogen function effect				
		4	6	8	10	12
		Diameters colonies (cm)				
At-1	2	0	2.18	0	0.43	0
	4	0	4.16	4.71	0.45	0
	6	0	6.23	5	0.47	0
At-2	2	0	3.33	2.81	1.6	0
	4	0	5.1	4.55	3.13	0
	6	0	7.83	6.23	2.41	0
At-3	2	0	2.8	1.83	1.68	0.8
	4	0	5	3.51	2.016	0.96
	6	0	6.51	4.33	2.65	1.36

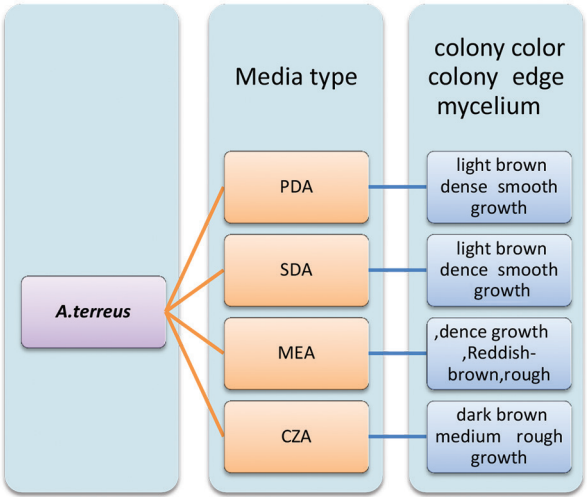

Figure 3 **Phenotypic characteristics of *A. terreus* on four media.**

Table 6. **Effect of different media on the growth of *A. terreus* isolates on PDA after 6 days of incubation at a temperature of 25 ± 2°C and pH 7**

		Culture media types			
		PDA	SDA	CZA	MEA
Isolates	Days	Diameters colonies (cm)			
At-1	2	3.18	2.25	2.1	1.28
	4	4.58	3	4.33	5.33
	6	5.75	7.13	6.23	6.36
At-2	2	2.85	3.43	2.83	1.5
	4	2.51	4.85	3.51	2.05
	6	4.7	8.65	7.86	2.76
At-3	2	2.25	2.66	3.83	1.66
	4	2.9	4.35	4.51	2.33
	6	3.56	7	6	2.8

LSD value for the type of center = 0.593.
LSD value for incubation days = 0.513.
LSD value for overlap = 1.781.

reached 8.65 cm while it was 7.86 cm on CZA. MEA that give colony diameter (2.76 cm) for isolate At-3, lower growth was recorded by PDA for isolate At-3 (3.56). It was found that the growth on SDA was 7 cm followed by culture media CZA (6 cm) at the end of incubation period while this ratio was 2.8 cm on MEA.

Discussion

There are a lot of researches that isolates of various fungi of the apples and recorded the proportion of the highest appearance and frequency of the *P. expansum*. These results were similar to those reached by Churki[13] in which he referred that *P. expansum* may record the highest rate displayed on apples which amounted to 100%, while *A. terreus* had the lowest emergence and fungi and other isolated from apples the lowest percentage of the appearance of 25%, while the current results do not agree with the findings of Pitt,[14] in which *Pencillium* ssp. comes second place after *Rhizopus stolinifer* in the damage apples. Molecular studies become crucial and necessary for identification of pathogenic fungi.[15] Based on the data reported[12] the molecular weight obtained in the current result agreed with them. On the other hand, the internal transcribed spacer (ITS) region of the fungal ribosomal DNA (rDNA) had been used as one of techniques for species identification because it is faster, has accurate species determination, specific, and are less feasible to be affected by exterior effects such as temperature changes and chemotherapy.[16] In some isolates which are not product gene (ITS) and does not appear, resulted that the isolates are able to link with DNA supplements appropriately to the absence of the relay so did not give a positive result.[17] There have been many studies that deal with environmental conditions, the temperature, pH of culture medium and type of culture medium which were the most important ecology factors that affect the growth of microorganisms and reproduction.[18] The degree of appropriate temperature for the growth of *A. terrues* ranging between 35–40°C[19] and in the current study founded that the best growth of the fungus was 30°C. While it was less growth in 15C°C. Increasing or decreasing of temperature higher or lower than optimum range may lead to breakdown of enzymes such as cellulose and was importance for the growth and mycotoxigenesis.[20] All isolates in this study are grown in pH 6. These results agree with the conclusion of Abubakar.[21] The pH effect on ions change which enter through cell membrane plasma had done at pH 5.5–6 if increased this value may lead to an imbalance that leading to weakness ions across, and the pH role of the exploits of the entry into force of ions across the membrane. pH becomes the cell membrane and adheres to hydrogen ions by blocking the passage of positive ions, either when the high pH, the hydrogen ions act to prevent the passage of negative ions.[23] The culture media SDA was found to be the most effective for supporting the maximum growth of *A. terrues*, because this medium contains nitrogen, potassium and phosphours which provided the fungi with necessary growth requirements.[24]

Conclusion

Number of fungi in apples were found and *A. terreus* was identified by PCR method and different ecology factors on growth fungus were studied. The best degree for the growth of isolates in the sixth day was 30°C. Maximum level of growth of *A. terreus* was at pH 6 for isolates in the sixth day and the growth rate for the same day on SDA reached 7.13 cm and was considered the best media for growth.

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