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Morphological and Molecular Identification of Fungi Isolated from Different Environmental Sources in the Northern Eastern Desert of Jordan

Sohail A. Alsohaili* and Bayan M. Bani-Hasan

Department of Biological Sciences, Al al-Bayt University, 25113 Mafraq, Jordan

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Abstract

This study is aimed at isolating and identifying filamentous fungi from different environmental sources in the northern eastern Jordanian Desert. The fungal species were isolated from soil and plant parts (Fruits and leaves). The samples were collected from different geographical locations in the northern eastern Desert in Jordan. The isolation of fungi from leaves and fruits was implemented by inoculating (1ml) from serial dilutions (10^{-3}- 10^{-6}) on Potato Dextrose Agar (PDA) plates. The plates were incubated at 28˚C for one week, then the fungal colonies were observed and pure cultures were maintained. The identification of fungi at the genus level was carried out by using macroscopic and microscopic examinations depending on the colony color, shape, hyphae, conidia, conidiophores and arrangement of spores. For the molecular identification of the isolated fungi at the species level, the extracted fungal DNA was amplified by PCR using specific internal transcribed spacer primer (ITS1/ITS4). The PCR products were sequenced and compared with the other related sequences in GenBank (NCBI). Eight fungal species were identified as: *Aspergillus niger*, *Aspergillus tubingensis*, *Alternaria tenuissima, Alternaria alternate*, *Alternaria gaisen*, *Rhizopus stolonifer*, *Penicillium citrinum*, and *Fusarium oxysporum*. The results showed that the *Aspergillus niger* was the most abundant fungus obtained from all the locations and resources, while the *Alternaria tenuissima* was the less prevalent one. It was also noticed that two of the *Alternaria* species colonized the leaves of plants at different locations. *Rhizopus stolonifer, Aspergillus tubingensis,* and *Fusarium oxysporum* were isolated and identified from all resources and locations.

Keywords: Filamentous fungi, Molecular identification, Internal transcribed spacer, *Aspergillus niger* survey, Jordan

1. Introduction

A Fungus is one of the most diverse microorganisms that inhabit different environmental sources such as soil, plant parts (leaves, root and fruits), water and food sources (Maheswari and Komalavalli, 2013, Sartori *et al.,* 2013; Rebecca *et al*., 2012). The growth and distribution of fungi are affected by different environmental factors such as temperature, pH, moisture, degree of aeration, amount and type of nutrients (Gaddeyya *et al*., 2012).

Soil fungi play an important and vital role in maintaining soil fertility and productivity, and are influenced by a number of factors, including soil properties and human activities (Bao *et al*., 2012). Fungi are very important organisms that inhabit the soil. They play an important part in nutrition and processes that lead to the improvement of the health and development of the plant (Mulani and Turukmane, 2014). However, the surface of the plant leaves and fruits is inhabited by several microorganisms, including filamentous fungi and yeast that cause the deterioration and spoilage of vegetables and fruits. This makes Fungi a high-priority concern because plants are food sources for consumers and are of great economic importance to farmers (Prabakaran *et al.,* 2011). Udoh *et al*., (2015) isolated a number of fungi species from some edible fruits and vegetables that were responsible for post-harvest spoilage of some edible fruits and vegetables. To solve these serious problems, there is still a need for the isolation and identification of filamentous fungi to have that under control, and prevent their damage of the agricultural crops.

The real number of fungi is still unknown; on the other hand, only 5-13 % of the overall evaluated worldwide fungal species have been characterized (Maheswari and Komalavalli, 2013). Thus, the fungal Isolation and identification from the different environmental sources is still very essential for the viewing and recognizing of more species, editing scientific classification, evaluating their effects in nature, and supplying strains for ecological remediation, biological control, and industrial aspects (Blackwell, 2011).

* Corresponding author. e-mail: : alsohaili@aabu.edu.jo.

The morphology of a fungal colony in filamentous fungi results from growing as fibers (hypha), that are cylindrical, threadlike 2–10 µm in diameter structures, long up to several centimeters, with different observations of colony features such as color, size, shape visible by the naked eye which was used classically to identify fungi (Lima and Borba, 2001). The morphology of fungi was observed under a compound microscope to examine the shapes forming from the arrangement of spores (Gaddeyya *et al.,* 2012). The morphological and biochemical identifications of fungi sometimes face many problems such as: the need for a great time, requiring high skill, and generating various morpho/biotypes within one species. The use of molecular identification is fast, sufficient, reproducible, and can provide high specificity to distinguish between the species and subspecies of fungi unlike the morphological and biochemical tests used in the laboratory diagnosis of fungi (liu *et al*., 2000; Sugita and Nishikawa, 2003).

Molecular identification techniques based on total fungal DNA extraction provide a unique barcode for the determination and identification of different fungal isolates up to a species level (Landeweert *et al.,* 2003). Molecular identification using this barcode has turned into an essential tool for mycologists studying fungal taxonomy, molecular evolution, population genetics or fungus-plant interactions (Moller *et al*., 1992). The identification of fungi using molecular techniques is carried out by the sequencing of PCR amplified part of 18S rRNA genes with universal primers to fungal species (Monod *et al*., 2005; Hensel and Holden, 1996).

The northern eastern Jordanian Desert is classified as a semi-arid area based on its climatic characteristics. The climate is hot in summers, but dry and chilly in winters. The rainy season extends from November to April with the rain fall being less than 200 mL/year (Abu Sada *et al*., 2015). On other hand, agriculture is the most important economical resource in this area due to the increase in farming activities over the last two decades. This area contains a large amount of groundwater used for the irrigation of plants. According to the knowledge of the researchers, there is no study dealing with the isolation and molecular identification of fungi from the northern eastern Jordanian Desert. This study is aimed at identifying fungi isolated from the soil, leaves and fruits of vegetation that exist in this arid environment. In addition, this study looks into the geographical distribution of the isolated fungi.

2. Materials and Methods

2.1. Collection of Samples

The soil and plant part (leaves and fruits) samples were collected from the northern eastern Jordanian desert in May of 2016. The samples were separated and labeled according to their location. The soil samples were collected from the plow layer (0–15 cm in depth) of the soil at different places. About 100 g of the soil were taken and packed into labeled sterilized bottles (Gaddeyya *et al.,* 2012). The leave and fruit samples were collected by cotton swabs from the leaves and fruits of different plant families, and were placed in sterile plastic bags (Rebecca *et al.,* 2012, Soni and Sharma, 2014).

2.2. Isolation of Fungi

The soil fungi were isolated by the soil dilution method. One gram of the soil sample was suspended in 10 ml of sterile distilled water to make serial dilutions (10^{-1} to 10^{-5}). One mL of each dilution was placed on Potato Dextrose Agar (PDA) containing 1 % streptomycin. The plates were incubated at 28˚C in the dark. The plates were observed for one week (Gaddeyya *et al.,* 2012; Reddy *et al.*, 2014). The leaves and fruit samples were placed and shaken in flasks filled with 100 mL of distilled water, then (0.2mL) of the sample was taken from the flasks and transferred into PDA medium with streptomycin. The cultures were incubated at room temperature in an incubator for three to five days. The fungal colonies were observed, and the pure cultures were maintained (Gaddeyya *et al.,* 2012; Javadi *et al*., 2012; Jasuja *et al*., 2013).

2.3. Macroscopic and Microscopic Examination of Isolated Fungi

The fungal morphology was studied macroscopically by observing the colony features (color, shape, size and hyphae), and microscopically by a compound microscope with a digital camera using a lactophenol cotton blue-stained slide mounted with a small portion of the mycelium (Gaddeyya *et al.,* 2012).

2.4. Molecular Identification of Fungal Species:

2.4.1. DNA Extraction and PCR Amplification

The DNA Extraction of genomic DNA from the fungi was conducted from a one-week-old PDA culture using DNeasy Plant Mini Kit (Supplied by QIAGEN). Primers (ITS1and ITS4) were used to amplify ribosomal internal transcribed spacer (ITS). PCR products were purified using the QIA quick PCR purification kit (Bao *et al*., 2012).

2.4.2. Sequencing and Analysis

The PCR products were sent for sequencing to Princess Haya Biotechnology Center, Jordan University of Science and Technology. The obtained sequences were compared with the other related sequences using BLAST search in GenBank (NCBI) (Liu *et al.,* 2000; Landeweert *et al*., 2003; Javadi *et al*., 2012).

3. Results

3.1. Macroscopic and Microscopic Features Isolated Fungi

In this study, the isolated fungi were examined on the basis of cultural, microscopic and morphological characteristics. Figure (1 – 8) show eight fungal species isolated and identified in this study

The colony morphology of *Aspergillums niger* shown in Figure 1 reveals a black color colony on the top (A1) and sulfur-yellow colonies on the reverse (A2), and the microscopic photograph shows (A3) the arrangement of conidia.

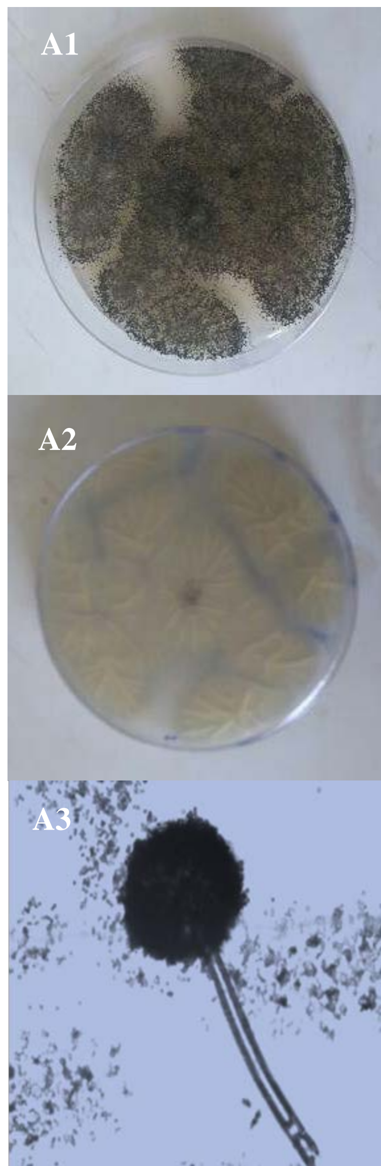

Figure 1. *Aspergillums niger* colony features on PDA (A1 Top, A2 Reverse) and conidia (A3).

Alternaria alternate features include a greenish-black surface on the top, black color on the reverse of the plate; the microscopic observed macroconidia are shown in Figure 2 (B1, B2 and B3) respectively.

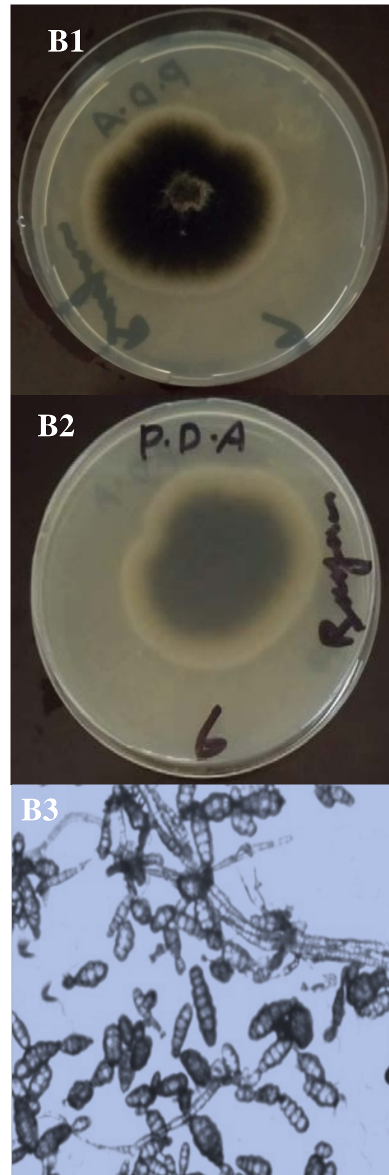

Figure 2. *Alternaria alternate* colony features on PDA (B1 Top, B2 Reverse) and chains of macroconidia (B3).

Figure 3 shows *Rhizopus stolonifer* with a deeply cottony texture of the colony having a white to gray-brown color on the top (C1), and pale white color on the reverse (C2) and dark pigment sporangium (C3).

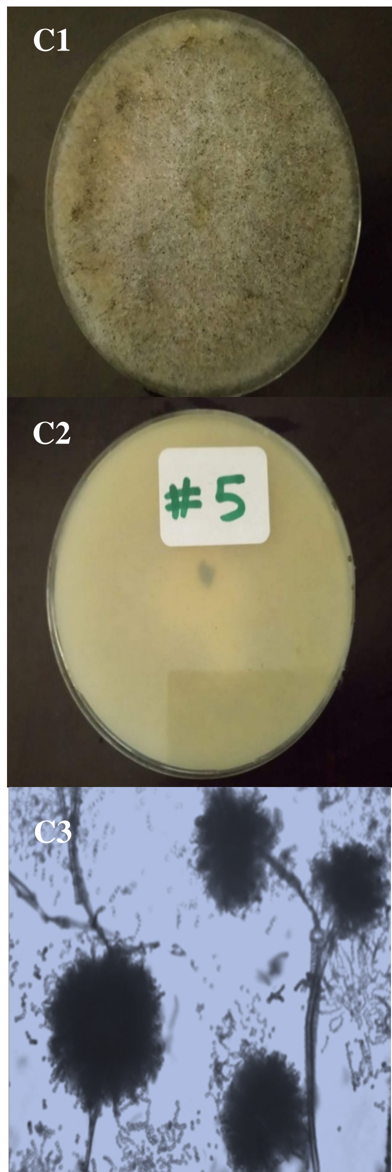

Figure 3. *Rhizopus stolonifer* colony features on PDA (C1 Top , C2 Reverse) and sporangium (C3).

The greenish-black surface on the top (D1), black color on the reverse of the plate (D2) and macroconidia of *Alternaria gaisen* (D3) as shown in Figure 4

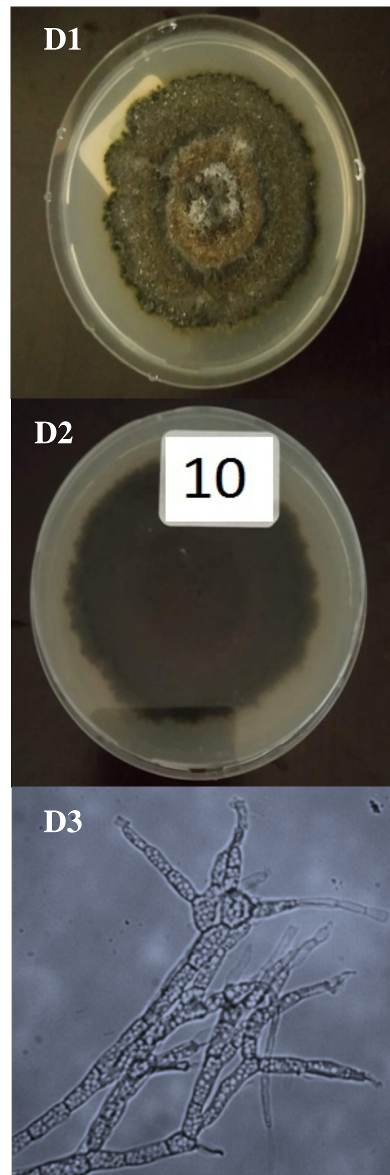

Figure 4. *Alternaria gaisen* features on PDA (D1 Top, D2 Reverse) and macroconidia (D3).

Penicillium citrinum morphological features are shown in Figure 5 with a bluish-green surface on the top (E1), pale yellow on the reverse (E2), and the brush arrangement of phialospores (E3).

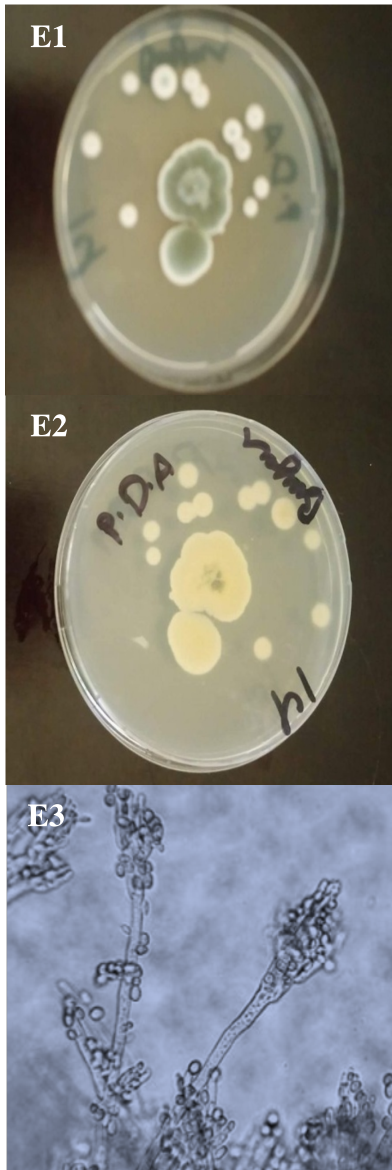

Figure 5. *Penicillium citrinum* features on PDA (E1 Top, E2 Reverse) and phialospores (E3)

The revealed morphological features of *Aspergillus tubingensis* include a white to pink color on the top (F1), light- yellow color on reverse (F2); finely wrinkled, globular, and warty conidia are shown in Figure 6.

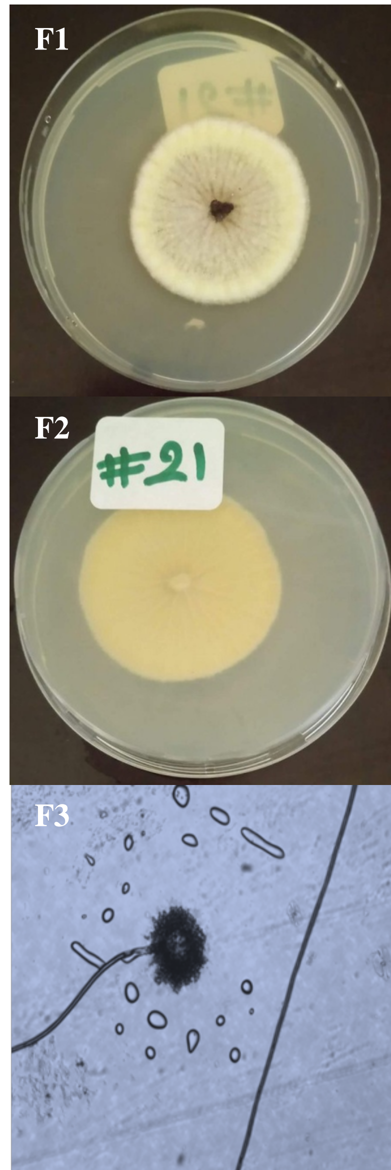

Figure 6. Plate Top, reverse and the conidia arrangement of *Aspergillus tubingensis* (F1, F2, and F3).

Figure 7 revealed the morphological characteristics of *Alternaria tenuissima* with greenish-black surface on the top (G1), black color on reverse (G2), and a microscopic photograph of the macroconidia (G3).

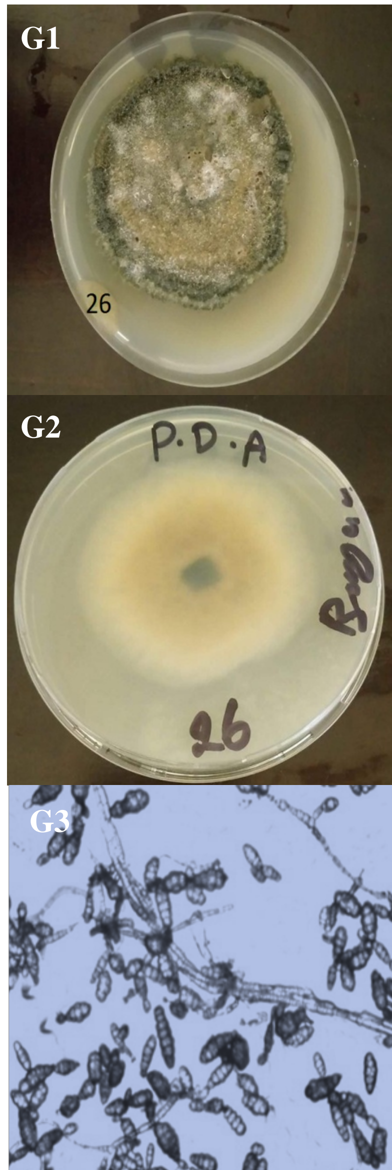

Figure 7. Plate Top (G1), reverse(G2), and the microscopic examination(G3) of *Alternaria tenuissima*.

The macroscopic identification of *Fusarium oxysporum* are shown in figure 8 (H1, H2) revealing a purple color on top, yellow color on reverse, while the microscopic photograph (H3) revealed sickle-shaped macroconidia.

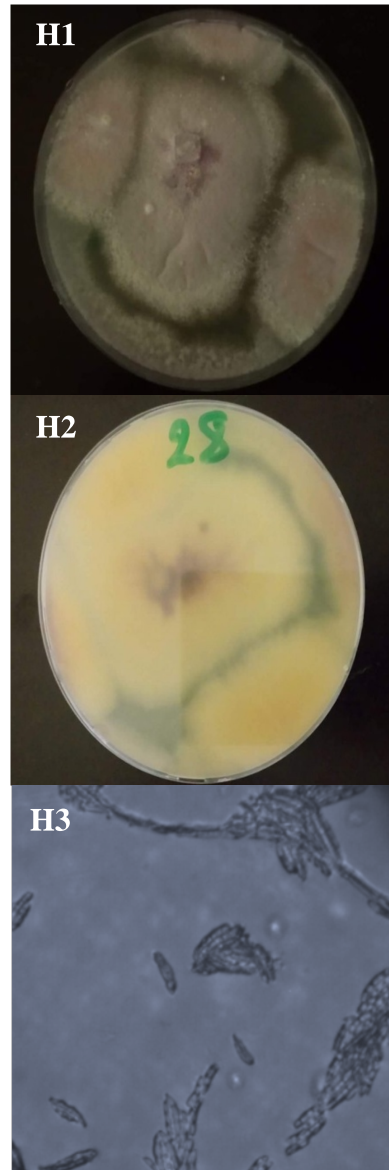

Figure 8. Plate Top, reverse and microscopic examination of *Fusarium oxysporum* (H1, H2, H3).

3.2. rDNA Sequences' Analysis

The ITS region of rDNA sequences are shown in table 1. Sequence analysis of the ITS regions of the nuclear encoded rDNA showed significant alignments of 97–99 % with the isolated fungal species.

Table 1. Identification of fungal isolates of ITS region of rRNA gene sequence.

Isolate	Species Identified	Length (bp)	Identity
BKSS1	*Aspergillus niger*	578	99%
BKSS2	*Alternaria alternate*	540	99%
BKSS3	*Rhizopus stolonifer*	731	97%
BKSS4	*Alternaria gaisen*	542	99%
BKSS5	*Penicillium citrinum*	478	97%
BKSS6	*Aspergillus tubingensis*	765	98%
[illegible]	[illegible]	[illegible]	[illegible]
BKSS8	*Fusarium oxysporum*	521	99%

3.3. Biodiversity of Fungal Isolates

The distribution of the isolated species from different sources is shown in table 2. The results showed that *P. citrinum* were isolated only from fruits, while *A. alternate* and *A. gaisen* were isolated from the leaves only, and *A. tenuissima* from the soil only, the remaining four species were isolated from all sources

Table 2. Distribution of isolated species at different sources.

Species/ Source	Fruits	Leaves	Soil
Aspergillus niger	+	+	+
Alternaria alternate	-	+	-
Rhizopus stolonifer	+	+	+
Alternaria gaisen	-	+	-
Penicillium citrinum	+	-	-
Aspergillus tubingensis	+	+	+
Alternaria tenuissima	-	-	+
Fusarium oxysporum	+	+	+

The distribution of the isolated species from different locations is shown in table 3. Eight species were isolated and identified. The result showed that *A. niger* was the most distributed species, and it was isolated from four locations, while *A. tenuissima* which was isolated from one location is the least distributed species.

Table 3. Distribution of Isolated Species at Different Locations**.**

Species/Location	Rasm Al-husan	Sabha	AL-salhiah	Al-bustaneh	Ruhbet Rakad
Aspergillus niger	+	+	-	+	+
Alternaria alternate	-	+	-	-	+
Rhizopus stolonifer	-	-	+	+	-
Alternaria gaisen	-	+	-	-	+
Penicillium citrinum	+	-	+	+	-
Aspergillus tubingensis	+	-	-	-	+
Alternaria tenuissima	-	-	-	+	-
Fusarium oxysporum	-	+	-	+	-

4. Discussion

This study was carried out to use various morphological and molecular examination methods to identify fungi isolated from the soil and plant parts (fruits, leaves) from the northern eastern Jordanian Desert. Eight fungal species were isolated and identified at the species level using rDNA ITS sequences comparison and analysis. The Isolated species belong to four classes as the following: Eurotiomycetes (*A. niger, A. tubingensis,* and *P. citrinum*), Dothideomycetes (*A. alternate, , A. gaisen,* and *A. tenuissima*), Sordariomycetes (*F. oxysporum*) and Mucoromycotina (*R. stolonifer*). Seven of these isolated fungi belong to ascomycetes, and one (*R. stolonifer*) belongs to zygomycetes. The high number and explosive dispersal of ascomycetes spore lead to a highly distributed species belonging to this phylum (Trail, 2007).

Five of the identified species (*A. niger, R. stolonifer, A. tubingensis, A. tenuissima, F. oxysporum*) were isolated from the soil. Soil is the most important source for the isolation of fungi, Chandrashekar *et al*. (2014) isolated and identified ten species belonging to three genera (*Aspergillus*, *Penicillium* and *Mucor)* from the rhizosphere soils in different agricultural fields of nanjangud taluk of the mysore district, karnataka, India. The results of another study conducted by Gaddeyya *et al*. (2012) revealed the isolation and identification of fifteen species belonging to six genera of fungi from the soil of agricultural fields at Salur Mandal, India. The variation and biodiversity of the isolated fungi from different geographical locations show different factors that affect the growth and distribution of fungi; these factors include soil pH, moisture content, salinity, organic carbon, nitrogen sulfur and potassium (Sharma and Raju, 2013; Yu *et al*., 2007).

Our current results showed the isolation and identification of five species from fruits (*A. niger, R. stolonifer, A. tubingensis, F. oxysporum,* and *P. citrinum*) and six species from leaves (*A. niger, A. alternate, R. stolonifer, A. gaisen, A. tubingensis,* and *F. oxysporum*) . The results came in agreement with the results of different studies that revealed the identification of the same genera

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of fungi from plant fruits and leaves (Udoh *et al*., 2015, Alwakeel, 2013; Kačániová and Fikselová, 2007). On other hand, the results of the current study disagreed with the results of Bashar *et al*. (2012) that showed the isolation of nine species of fungi belonging to eight genera of filamentous fungi from the leaves and fruits of the breadfruit plant. The variations of the isolated and identified fungal species refer to the variations of plant type and environmental factors previously mentioned.

In this study the fungal isolates were firstly identified to a genus level using a morphological examination depending on the colors of colony formed at both sides, the top and reverse of the fungal cultures. The microscopic examination of the shape of the spore-producing structures was used for further identification. The morphological examination and identification of fungi are useful for the identification of isolates up to the family or genus level (Wang *et al*., 2016). However, this identification is not enough to identify the isolated fungi up to the species level (Lutzoni *et al*., 2004).

The molecular identification was carried out by DNA barcoding using the ITS region sequencing. The ITS rDNA sequences were compared to those in the databases using NCBI-BLAST. Eight species were identified using DNA barcoding with an identity range between 97 – 99 %. It is also proposed that ITS rDNA region sequence is one of the most important tools for the identification of the fungal species isolated from environmental sources (Anderson and Parkin, 2007); hence, it has been widely used to detect the soil fungal community, and as an improvement of the classical identifications. ITS rRNA genes are excellent candidates for the phylogenetic analysis because they are universally distributed, functionally constant, sufficiently conserved, and of adequate length to provide a deep view of evolutionary relationships (Madigan *et al*., 2012).

According to the results of this study, the biodiversity of the isolated fungal species is clearly observed among the geographical locations. The distribution and abundance of the fungi differ from one isolation location to another. Some species such as the *A. niger* were isolated and identified from four regions and locations, whereas *A. tenuissima* was isolated only from one region. The biodiversity of fungi refers to the physiochemical properties of the environment such as; PH of the soil, temperature, and humidity while taking into consideration that all locations belong to the desert environment.

5. Conclusion

The isolation and identification of filamentous fungi from the northern eastern Jordanian Desert displayed the presence and abundance of some economically-important fungi. As this study is the first of its kind in Jordan in that it specifically used the molecular technique which added great benefits to the process of distinguishing between similar species of fungi in comparison with the classical techniques. There is a large variation in the distribution of fungal species in different geographical locations and also from different source of isolation. Therefore, this study recommends further work to be done in the future to isolate and identify more of the filamentous fungi for taxonomy and pathogenicity investigations. It also recommends studying other types of fungi such as mycorrhiza and yeast belonging to the northern eastern Jordanian Desert.

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