Identification of Different Fungal Fruit Rot Pathogens of Date Palm *(Phoenix dactylifera* L.) using ITS and RAPD Markers.

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Abstract

The fungal diseases of date palm (*Phoenix dactylifera* L.) are one of the most serious pathogens attacking different parts of date palm trees and causing significant reduction in date palm growth; development and production. Recently, the infection of date palm fruit with rot disease in Basra province/ Iraq is raising concern for the workers in dates production. The goal of present study was to isolate and identify these pathogens using molecular approaches. Four genera were isolated from infected Barhee cv fruits which were: *Alternaria*; *Cladosporium*, *Epicoccum* and *Ulocladium*, most abundantly genus was *Alernaria* (35%), followed by *Cladosporium* (25%). The high virulence affects of these pathogens were approved at laboratory on dates fruits. Molecular identification using internal transcribed spacer primers (ITS1 and ITS2) were performed, ITS DNA sequences of each examined fungi were applied into BLAST (NCBI) and the species identities were proven as *A. tenuissima*; *C. herbarum*; *E. sorghinu*, and *U. atrum*. Further, five different RAPD markers were applied to show the genetic variation among these pathogens, results showed that dendrogram cluster analysis elucidated a genetic distance of 83-94% among fungal species, which separated into different clusters. Additionally, the observations of extracellular enzyme activity of examined fungi were followed, results showed a good activity of both cellulase and protease enzymes, but no lipase activity was observed with all tested fungi.

Keywords: Enzyme activity; Fungi; Pathogenicity; Plant diseases; Molecular approaches.

1. INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is one of the family Arecaceae , a dioecious, perennial, monocotyledonous tree cultivated mainly for their nutritive fruits (Abass, 2013a), dates fruits are a good source of energy and are rich in nutrients, their fruits consist of 70% carbohydrates; mostly as sugars, and 15-30% water. Dates are also a source of different minerals including iron, potassium and calcium, with low levels of sodium and fat (Thabet et al., 2010, Dayani et al., 2012). Additionally, date palm tree play an important role in the sustainable agriculture in many countries around the world with a long history of cultivation and utilization in Middle East and north Africa for more than five thousands years (Al-Khayri, 2007) .

Diseases of Date palm are rising as a serious concern during the last decades. The fungal pathogens of date palm are considered problematic agents and lead to significant reductions of growth, development, and production of date palm around the world (Abass et al., 2007, El-Hassani et al., 2007). Several fungal pathogens has been identified on date palm host such as *Fusarium oxysporum*, the causal agent of Bayoud (El-Hassani et al., 2007), *Phytophthora* sp., the causal agent of Belaat (Howard and Carpenter, 1993); *Ceratocystis paradoxa*, the causal agent of bending head (Elliot et al., 2004), species *of Alternaria*, *Graphiola*, *Pestalotia*, *Mycrosphaerella* and *Phoma* are the causal agents of leaf spots (Sheir et al., 1982; Abass et al., 2007).

Date palm fruits spot disease is an important disease and infect dates both at field and markets, the high water content of fruits (up to 30%) with high nutritive value are making them a good target for invading with many fungi such as *Aspergillus*, *Alternaria*, *Cladosporium*, *Eurotium*, *Fusarium*, *Mucor*, *Mycosphaerella*, *Penicillium* and *Rhizopus* (Djerbi, 1983; Gherbawy, 2001). In Basra province, majority of fungal diseases have been concentrated in the date palm orchards nearest to the river banks, such as Shaat-Al-Arab where the high level of humidity could contribute to the spread of these fungal infections.

Recently, the advances in molecular biology approaches have enabled the microbiologists to reveal the polymorphism on DNA levels by utility of different DNA markers, especially after the introducing of polymerase chain reaction (PCR) technique. Several efficient protocols have been achieved in microbial taxonomy using DNA markers, including ITS (internal transcribed spacer) which is coding for ribosomal RNA gene complex with a great importance in fungal identification; including 16S, 5.8S and 28S fungal rRNA subunits, those are separated by ITS sequences. Noteworthy, ITS markers are amplifying the highly variable region flanked by these primers that encompass the 5.8S coding sequence and situated between small and large subunit coding sequence of the ribosomal operon (Freifelder, 1983; White et al., 1990). ITS sequences have been applied successfully in fungal identification with different fungi such as *Alternaria*, *Aspergillus*, *Penicillium* and *Nigrospora* (Pashley et al., 2012, Abass, 2013b; Nilsson et al., 2014; Visagie et al., 2014).

Another technique for genetic diversity detection among plant pathogenic fungi is RAPD (randomized amplified polymorphic DNA) which employed in several studies due to many advantages such as high reproducibility and variability results without any need for knowledge of DNA sequence; cost-effective technique and using very small amount of DNA (Huang et al., 1997; Pryor Michailides, 2002). Different genetic variations have been revealed using RAPD markers in fungal plant pathogens such as *Alternaria*, *Aspergillus*, *Dreshlera*, *Fusarium*, *Mucor*, *Penicillium* and *Rhizopus* (Gupta et al., 2010; Kakvan et al., 2012; Akhtar et al., 2013).

The present study has been conducted to identify the fungal species pathogens of date palm fruit spot disease using molecular approaches.

2. Materials and Methods

2.1 Fungal Isolation

Infected date palm fruits of Barhee cultivars, with evident symptoms of fruit spot, were collected from different areas in Basra province and brought to the laboratory. The isolation procedure of Keith *et al.* (2006) was followed. The symptomatic fruits tissues were sectioned into small pieces, approximately 1–2 cm^2, and surface-sterilized by dipping in a 10% solution of sodium hypochlorite (as 1% commercial

Chlorox) for 5 min and rinsed several times in distilled water, and placed on the surface of potato dextrose agar supplemented with 150 mg/ L chloramphenicol and kept at 25 ˚C for several days. The isolates of the fungus were kept as single spore transfers according to Vitale and Polizzi (2005), all fungal samples were characterized to genus level according to Ellis (1971). Isolation frequencies of fungal genera were calculated.

2.2 Pathogenicity Test

Healthy date palm fruits at Rattab stage were used in this experiment. Date fruits were detached from orchard- grown trees and brought to the laboratory, a sterilization with 10% solution of sodium hypochlorite was followed, fruits dried on sterilized filter paper. Further separation of the fruits was done into two set of groups; first group was wounded at the centre with sterile needle on a depth of 1 mm and infiltrated with 10 µL of each conidial suspension (10^6 cfu/ mL), second group was left unwounded and sprayed with fungal conidia suspension. Treated and control fruits were kept at growth chamber (25 ˚C and 90% RH). Fruit spot development was visualized 9 days post-inoculation on the basis of lesion size. The experiment repeated twice for results confirmation. The reisolation of the pathogen from the inoculated fruits was done on PDA plates as mentioned above to fulfill Koch's postulates.

2.3 ITS Markers Analysis

2.3.1 Fungal DNA Extraction

The procedures of Zolan and Pukkila (1986) were followed for fungal genomic DNA extraction, purification and ethanol precipitation. Briefly, single-spore cultures were placed on Potato Carrot Agar (PCA) medium at 25 °C for 7 days. The mycelium and conidia were collected (approximately 10 g) and ground with liquid nitrogen at room temperature, then extracted with 600 µL extraction buffer [1% hexadecyltrimethylammonium bromide, 0.7 M NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% 2- mercaptoethanol], vortexed and incubated at 60 ° C for 30 min. An equal volume of chloroform:isomyl alcohol (24:1, v/v) was added, tubes were then centrifuged 5 min at 13000 rpm. The aqueous phases were recovered into fresh tubes containing isopropanol and followed by a second centrifugation for 1 min. The DNA pellets were resuspended in 300 µL of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. DNA concentrations for all samples were quantifies by Nano-Drop

spectrophotometer (Bio-Rad, USA) at A260/280, gDNA templates were diluted to 30 ng/ µL with TE buffer.

2.3.2 ITS Markers and PCR Amplification

Universal primers (ITS1 and ITS4) were selected for molecular identification of fungal species. The sequences of primers were: ITS1: 5':TCCGTAGGTGAACCTGCGG-3', which hybridizes at the end of 18S rDNA and ITS4: 5':TCCTCCGCTTATTGATATGC-3', which hybridizes at the beginning of 28S rDNA (White et al., 1990). The Polymerase Chain Reaction (PCR) was carried out in 0.2-mL polypropylene tubes with a total mixture of 50 µL consisting of a 4 ng of gDNA template, 5 µL of 10×polymerase buffer, 8 µL of dNTPs (1.25 mM), 1 µL of *Taq* DNA polymerase (Roche) and 1 µl of each primer, and distilled water up to 50 µL.

The thermal cycler used was equipped with a heated lid (M. J. Research Inc., Waltham, Massachusetts, USA). The PCR cycle was set up as follow: 5 min initial denaturation and enzyme activation at 95 °C, followed by amplification for 35 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 10 min (Abass and Muhammed, 2014).

2.4 RAPD Markers and PCR Amplification

Five different Decamer nucleotide primers manufactured by Bioneer-Korea were used for RAPD analysis (Table 1, marker description). Each polymerase chain reaction (PCR) was carried out as mentioned in *2.3.2*. Amplification was performed in a thermal cycler using the following conditions: denaturation at 95 ºC for 3 min; 35 cycles of 1 min denaturation at 94 ºC, 1 min annealing at 36 ºC and 2 min extension at 72 ºC; and a final extension at 72 ºC for 7 min. All PCR reactions were run on Agaorse gel (1.2%) for DNA profile.

The PCR products were sequenced and analyzed by comparison with all available sequences in the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the Basic Alignment Sequence Tool (BLAST): (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

254 Data Analysis

DNA marker of Thermo Fisher Scientific (100-1500 bp) was used to detect the precise molecular sizes of each individual fragments using the PhotoCapt MW software 10.0 (Vilber Loumart) and photographed under UV light. Total bands number, polymorphic bands and band lines were scored visually. All bands were visually scored as present (1) or absent (0) to create the binary matrix. Only clear and reproducible amplified bands were considered for estimation the genetic similarity coefficient and distance for all pairs of treatments according to Nie and Li (1979), as fellow:

$$\text{Genetic Similarity Index} = 2A/(B + C)$$

Where, (A) number of similar bands in both treatments, (B) and (C) total number of bands in the first and second treatments.

The similarity coefficients were used to construct a dendrogram illustrating genetic relationship using the unweighted pair group mean average (UPGMA) method (Sneath and Sokal, 1973).

The following primers parameters were measured as follow (according to Alansari et al., 2015):

$$\text{A- Primer efficiency (\%)} = \frac{\text{the total number of bands amplified by primer}}{\text{the total number of obtained bands}} \times 100$$

$$\text{B- Polymorphism (\%)} = \frac{\text{the number of polymorphic bands}}{\text{the total number of bands amplified by the same primer}} \times 100$$

Table 1. RAPD primer sequences and GC%.

Primer Code	Primer sequence 5'----------3'	GC%
OPR3	ACACAGAGGG	60
OPR11	GTAGCCGTCT	60
OPR14	CAGGATTCCC	60
OPR19	CCTCCTCATC	60
OPR20	ACGGCAAGGA	60

2.5 Extracellular Enzyme Analysis

2.5.1 Cellulase Activity

All fungal species on were grown YEPA , containing : 0.1 g yeast extract, 0.5 g peptone, 16 g agar in 1 liter of distilled water, and supplemented with 0.5% (w/v) N-carboxymethyl cellulose. Each plate was incubated at 25 ° C. The plates (9 cm diameter) were flooded with 5 mL of Congo red (0.1%) and then destained with sodium chloride (1%) for 15 min. The clear zones around the colonies were measured by taking the average of three directions on each Petri dish.

2.5.2 Protease activity

The protease activity was assayed following the procedures described by Amirrita et al. (2012) on GYPA medium containing: 1 g glucose, 0.1 g yeast extract, 0.5 g peptone, 16 g agar in 1 liter of distilled water, amended with gelatine (0.4% w/v). Both GYPA and gelatine were sterilized separately by autoclaving for 20 min. Saturated aqueous of ammonium sulphate was used (5 mL/ plate) to flood the cultures. The saturation of ammonium sulphate was done by dissolving a 75 g of ammonium sulphate in 100 mL of distilled water. The clear halo around the colonies indicating the proteolyitc activity and was measured by taking the average of three directions on each Petri dish.

2.5.3 Lipase activity

The procedure of Sierra (1957) was followed to determine the lipase activity. Briefly, the medium of Peptone Agar Medium (PAM) containing: 10 g peptone, 5 g NaCl, 16 g agar in 1 liter of distilled water) supplemented with sterilized Tween 20 at 1% (v/v) was inoculated with fungal colony plugs of 0.5 cm of tested species and incubated at 25º C. The clear halo indicating the lipase activity.

2.6 Statistical Analysis

Pathogenicity experiment was employed according to CRD design (completely randomized design) with ten replicates for each treatment, statistical significance was confirmed by ANOVA analysis and with revised least significant difference test at 0.01. The pathogenicity results were expressed as mean and standard deviation of the mean.

3. RESULTS AND DISCUSSION

3.1 Isolation of Fungi

Different fungi associated with date palm fruit deterioration were isolated, a total of four fungal genera were identified on the basis of their morphological (on PDA medium) and microscopic characteristics which were *Alternaria*, *Cladosporium*, *Epicoccum* and *Ulocladium* (**Fig. 1**), the highest isolation frequency was observed with the fungal *Alternaria* (35%), followed by *Cladosporium* (25%), while the lowest frequency was obtained with *Ulocladium* (18%) (**Fig. 2**). The high abundance of *Alternaria* spp. (*the Hyphomycetes* in the *Fungi Imperfecti*), could be explained by their cosmopolitan ability includes saprophytes on organic substrates and parasites on living plants (Rotem, 1994; Thomma, 2003), in way enable these species to grow in soil, plants, as well as in the air (Shelton et al., 2002).

The current results are in a good agreement with many other published researches showed the isolation of these fungi and their association with fruit rot on different plant hosts such as date palm, pistachio and citrus fruits (Gherbawy, 2001; Pryor and Michailides, 2002; Akhtar et al., 2013), additionally, these fungi are well known as true pathogens on date palm and caused leaf spot disease (Carpenter and Elmer, 1978; Kassim et al., 1983; Abass et al., 2007).

3.2 Pathogenicity test results

The results of pathogenicity test on date palm fruits of Barheee cultivar revealed the virulence of *Alternaria* genus, which was obvious in both wounded and unwounded treatment, the average of lesion size were 4.14 and 2.37 mm, respectively, with high significant differences than other fungi, followed by *Ulocladium* which reported the values of 1.97 and 1.75 mm, as lesion sizes in wounded and unwounded treatments, respectively, without any significant differences than those observed at *Epicoccum* and *Cladosporium* treatments (**Fig. 3**).

The high level of pathogenicity in the artificial inoculation with *Alternaria* and the examined fungi on date palm fruits could be attributed to their enzymatic and toxic activity. Several toxins have been isolated and identified from the culture filtrate of *Alternaria* (both host-specific and nonspecific toxins) such as, alternariol (AOH);

alternariol monomethyl ether (AME); AM-toxins; AF-toxins; AK-toxins and ACT-toxins (Soma et al., 2011; Scott et al., 2012; Tsuge et al., 2013), additionally, these pathogenic fungi are well known for their enzyme activity, more specifically, the role of extracellular degradative enzymes in disease progress, including protease and cellulase (Abass, 2005; Hameed and Abass; 2006, Abass and Muhammed, 2014).

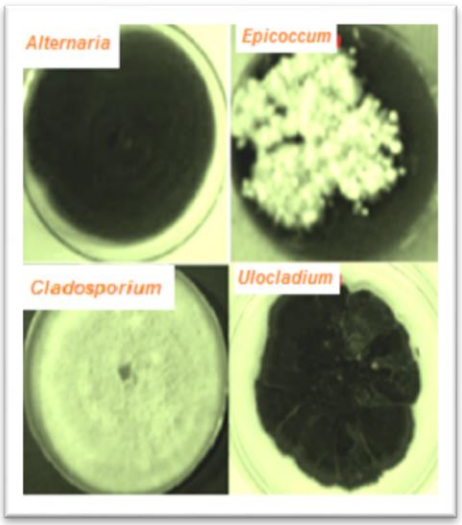

Figure 1. Pure cultures of *Alternaria*¸ *Cladosporium* , *Epicoccum* and *Ulocladium* on **PDA** plates.

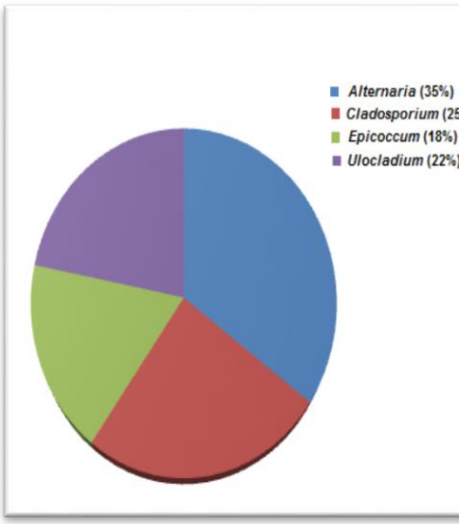

Figure 2. Isolation frequencies of different fungi associated with dates fruits rot disease.

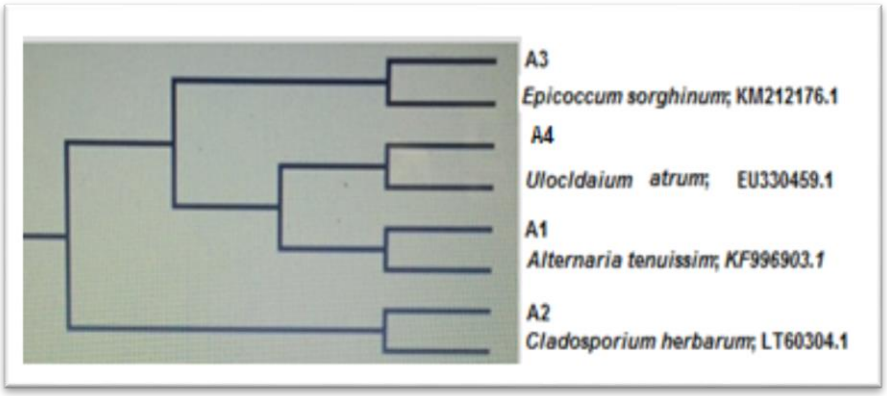

Figure 4. Phylogenetic tree of studied fungi isolated from dates fruits with their identical according to BLAST results. A1: *Alternaria*; A2: *Cladosporium*; A3: *Epicoccum*; A4: *Cladosporium.*

3.3 Molecular Characterization Using ITS Sequence

The genomic DNA of isolated fungi from date palm fruits were extracted and employed in PCR amplification using ITS1 and ITS4 primers as described by White et al. (1990), the sequence analysis of ITS regions of the nuclear encoded rDNA showed significant alignments of 98–99% among studied fungi according to the BLAST search. The results revealed the identity of each fungus, as fellow: *Alternaria tenuissima* with un identity of 98% (KF996903.1); *Cladosporium herbarum* with un identity of 99% (LT60304.1); *Epicoccum sorghinum* with un identity of 98% (KM212176.1) and *Ulocladium atrum* with un identity of 98% (EU33049.1) (**Fig. 4, Table 2**).

Molecular identification of pathogenic fungi has received attentions by many microbiologists, different approaches were employed to identify fungal species such as AFLPs; RAPDs; SNPs and ITS sequences (Roeckd-Drevet et al., 1997; Komjati et al., 2008; Martin, 2008; Soma et al., 2011; Alwakeel, 2013; Abass, 2013b; Abass and Muhammed, 2014). The Internal transcribed Spacer (ITS) regions of ribosomal DNA (rDNA) has a great importance in fungal identification; both ITS primers ITS1 and ITS4 were used to amplify these regions which compass the 5.8S coding sequence situated between large and small units (White et al., 1990). **Fig. 4** depicts the phylogenetic tree generated based on the similarity of ITS sequences of the examined fungi as well as their similar matching obtained from GenBank, cluster analysis shows that the studied fungi were clustered in four different groups aligned with their identical match.

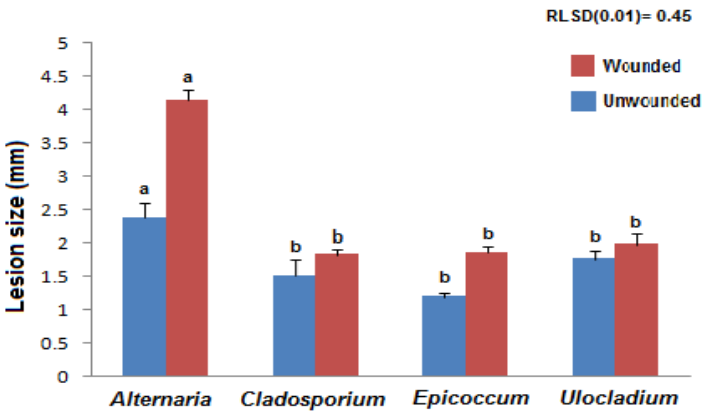

Figure 3. Pathogenicity test results of *Alternaria¸Cladosporium, Epicoccum* and *Ulocladium* on **Barhee** fruits.

Table 2. ITS sequences and alignments of studied fungi isolated from dates fruits.

***Alternaria* :** ITS sequences has been submitted into GenBank and under the gene accession number **: KX 981205** *

```
   1 tccgtaggtg aacctgcgga gcacattgcg ccctttggta ttccaaaggg ggaacctctc
  61 ggggttacag ccttgctgaa ttattcaccc ttgtcttttg cgtacttctt gtttccttgg
 121 tgggttcgcc caccactagg acaaacataa acctttttgta attgcaatca gcgtcagtaa
 181 cgagcgtcat ttgtaccctc tcaacaacgg atctcttggt tctggcatcg atgaagaacg
 241 cagcgaaatg cgataagtag tgtgaattgc agaattcagt gaatcatcga atctttgaac
 301 gcacattgcg ccctttggta ttccaaaggg catgcctgtt cgagcgtcat ttgtaccctc
 361 aagctttgct tggtgttggg cgtcttgtct ctagctttgc aggagactcg ccttaaagta
 421 attggcagcc ggcctactgg tttcggagcg cagcacaagt cgcactctct atcagcaaag
 481 gtctagcatc cattaagcct ttttttcaac ttttgacctc ggatcaggta gggatacccg
 541 ctgaacttaa gcatatcaat aagcggagga
```

***Cladosporium* :** ITS sequences has been submitted into GenBank and under the gene accession number**: KX985926**

```
1 gacctgcgga gggatcatta caagaacgcc cgggcttcgg cctggttatt cataaccctt

 61 tgttgtccga ctctgttgcc tccggggcga ccctgccttc gggcgggggc tccgggtgga
121 cacttcaaac tcttgcgtaa ctttgcagtc tgagtaaact taattaataa attaaaactt
181 ttaacaacgg atctcttggt tctggcatcg atgaagaacg cagcgaaatg cgataagtaa
241 tgtgaattgc agaattcagt gaatcatcga atctttgaac gcacattgcg ccccctggta
301 ttccgggggg catgcctgtt cgagcgtcat ttcaccactc aagcctcgct tggtattggg
361 caacgcggtc cgccgcgtgc ctcaaatcgt ccggctgggt cttctgtccc ctaagcgttg
421 tggaaactat tcgctaaagg gtgttcggga ggctacgccg taaaacaacc ccatttctaa
481 ggttgacctc ggatcaggta cgggcttcgg cctggttatt cataaccctt tgtgaattgc
```

***Epicoccum*:** ITS sequences has been submitted into GenBank and under the gene accession number**: KX985927**

```
  1 ttcgtaggtg acctgcggag gatcattacc tagagttgta ggctttgcct gctatctctt
 61 acccatgtct tttgagtacc ttacgtttcc tcggtgggtt cgcccaccga ttggacaaat
121 ttaaaccctt tgcagttgaa atcagcgtct gaaaaaactt aatagttaca actttcaaca
181 acggatctct tggttctggc atcgatgaag aacgcagcga aatgcgataa gtagtgtgaa
241 ttgcagaatt cagtgaatca tcgaatcttt acccatgtct tttgagtacc ttacgtttcc
301 ggggcatgcc tgttcgagcg tcatttgtac cttcaagctc tgcttggtgt tgggtgtttg
361 tctcctgtag actcgccttа aaacaattgg cagccggcgt attgatttcg gagcgcagta
421 catctcgcgc tttgcactca taacgacgac atccaaaagt acatttttac actcttgacc
481 tcggatcagg tagggatacc
```

***Ulocladium*:** ITS sequences has been submitted into GenBank and under the gene accession number**: KX985928**

```
   1 gcanggctgg catccttcgg ggtatacacg ccntntgaca tgnaattatt cacccgtgtc
  61 ttttgcgtac ttcttgtttc cttggtgggt tcgcccacca taggacaaac cataaacctt
 121 ttgtaattgc aatcagcgtc agtaaaaaaa ttaataatta caacttttaa caacggatct
 181 cttggttctg gcatcgatga agaacgcagc gaaatgcgat aatcagcgtc agtaaaaaaa
 241 ttcagtgaat catcgaatct ttgaacgcac attgcgccct ttggtattcc aaagggcatg
 301 cctgttcgag cgtcatttgt accctcaagc tttgcttggt gttgggcgtc ttgtctccag
 361 ttcgctggag actcgccttа aagtaattgg cagccggcct actggtttcg gagcgcagca
 421 caagtcgcgc tctcttncag ccaaggtcag catccacaaa gccttttttc aacttttgac
 481 ctcggatcag gtagggatac ccgctgaact ccntntgaca tgnaattatt cacccgtgtc
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*https://www.ncbi.nlm.nih.gov/WebSub/

3.4 RAPD Marker Analysis

RAPD technique have been applied to detect further genetic variations among examined fungi, three RAPD primers (OPAR3; OPAR11 and OPAR20) were considered in data analysis, revealing to be polymorphic and reproducible among studied species. A total of 63 amplified bands were scored for all primers, 49 bands were polymorphic among the species profile, with a polymorphism percent of 80% (**Table 3)**. High informative profile was found with primer OPAR11, a total of 17 polymorphic and 4 monomorphic bands were scored (**Fig. 5**), with the highest polymorphism percentage (80.95%), followed by the primer OPAR20 which amplified 19 polymorphic bands and 5 monomorphic bands, with a polymorphism percent of 79.15%. Regarding the primer efficiency, results showed that the highest efficiency was observed with the primer OPAR20 and reached 38%, while the lowest one was seen in primer OPAR3 (28.5%).

Regarding the results of similarity indices (shown in **Table 4**), RAPD profile analysis revealed that all studied fungi had high level of genetic variations (0.06-0.17) according to Nei' and Li's index, furthermore, the UPGMA dendrogram of RAPD patterns was computed (**Fig. 6**), and indicated that all examined fungi were separated into different clusters with the highest average of genetic distances (0.94-0.83) among them. Herein, the results of this experiments showed an evident and high productivity of RAPD technique for confirming the genetic variations among four different genera of fungal fruit rot pathogens.

The sensitivity of RAPD technique in detection of genetic variations among fungi which approved in this study is in accordance with many studies showed the reliability and reproducibility of RAPD technique in identification of genetic variations (Huang et al., 1997; Dini-Andreote et al., 2009; Gupta et al., 2010; Kakvan et al., 2012; Akhtar et al., 2013).

Regarding the differences in RAPD markers efficiencies and polymorphism which were obtained in this results, thus, could be related to their sequence differences in way affect their sensitivity and binding activity across the fungal genomic DNA (Arnau et al., 1994; Abass, 2013b).

Table 3. RAPD primers with total number of bands, polymorphic bands, primer efficiency, polymorphism percentage.

RAPD code	Polymorphic bands	Total No. bands	Primer efficiency	Polymorphism %
OPAR3	13	18	28.50	72.20
OPAR11	17	21	33.30	80.95
OPAR20	19	24	38.00	79.15
Total	49	63		

Table 4. Similarity indices of Nie' and Li's coefficients of date palm fungal fruit spot pathogen obtained from RAPD analysis.

Fungus	*A. tenussima*	*C. herbarum*	*E. sorghinum*	*U. atrum*
A. tenussima	1			
C. herbarum	0.160	1		
E. sorghinum	0.150	0.110	1	
U. atrum	0.120	0.060	0.170	1

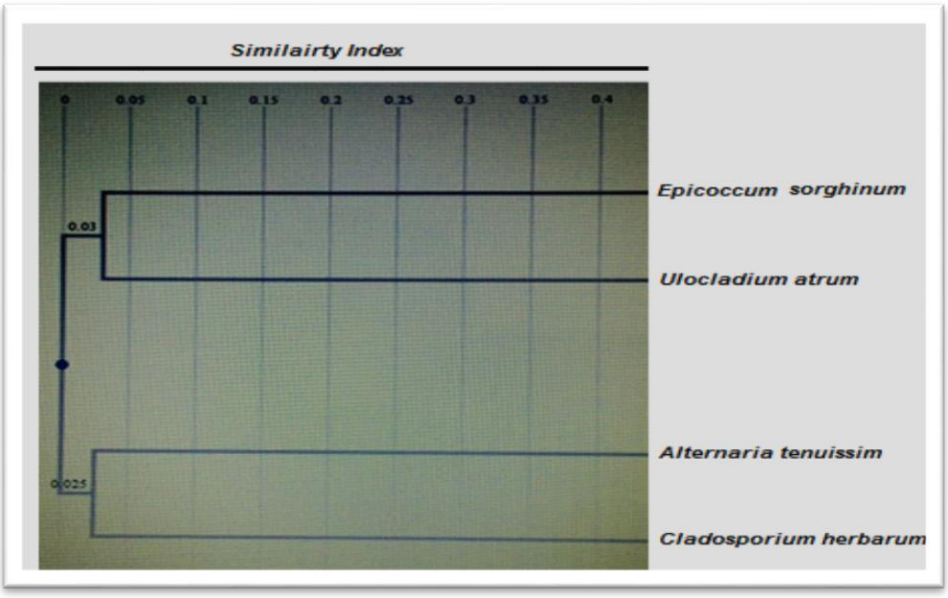

Figure 6. Dendrograms generated by UPGMA cluster method based on RAPD Markers of different date palm fruits rot fungal pathogens.

3.4 Extracellular Enzyme Activity

The results analysis of extracellular enzyme activity showed that the best fungal genera for cellulase activity were *Alternaria* and *Ulocladium*, and reported the values of 20 mm, as an enzyme activity, followed by *Cladosporium* and *Epicoccum* (**Table 5**), in terms of protease activity, the genus of *Alternaria* showed the highest activity (20 mm), followed by *Cladosporium* genus (15 mm). The production of hydrolytic enzymes by fungal pathogens is a crucial factor in pathogenicity, several studies showed their involvement in different biological functions including host specificity, deterioration of food, and decomposition of organic matter (Ahmed et al., 2006). The highest activity of cellulase and protease in *Alternaria* could be one the explanations of their virulence effect that observed in pathogenicity experiments. The positive results of *Cladosporium* and *Epicoccum* are in accordance with many published researches revealed them as good producers of cellulase and protease enzymes (Favaro et al., 2011; Agnes et al., 2015).

No detection of lipase activity was observed in all studied genera, regardless the time of incubation using Tween 20 at 1%, several studies showed the suitability of Tween 20 as a substrate in fungal lipase assay in solid medium (Tan et al., 2004; Amirrita et al., 2012; Agnes et al., 2015), the negative results of lipase activity have been observed in many fungal genera such as *Curvularia*, *Fusarium*, *Thialoviopsis* and *Trichoderma* (Abass, 2005; Agnes et al., 2015).

Table 5. Extracellular enzyme activity of date palm fruit rot pathogens.

Fungal Species	Cellulase Activity (mm)			Protease Activity (mm)			Lipase Activity (mm)		
	EA*	ZD	RG	EA	ZD	RG	EA	ZD	RG
A. tenuissima	+	20	65	+	20	60	-	-	55
C. herbarum	+	15	50	+	15	50	-	-	40
E. sorghinum	+	15	55	+	10	45	-	-	35
U. atrum	+	20	60	+	10	40	-	-	40

Where*: EA: enzyme activity; ZD: zone diameter; RD: radial growth; EA: enzyme activity.

1.5 Conclusions

Herein, the obtained results indicated the isolation and molecular identification of four different fungal genera associated with date palm fruit rot disease, ITS DNA sequencing revealed their identities on species level as *Alternaria tenuissima Cladosporium herbarum*, *Epicoccum sorghinum* and *Ulocladium atrum*. Pathogenicity experiments on Barhee cultivar fruits and extracellular enzyme activity showed the virulence affects of studied fungi. Further investigations are required on aspects of toxicology and histology on these fungi to improve our understanding for their pathogenicity on date palm fruits.

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