First Report on Four Casual Agents of Leaf Spot Diseases and the Resistance Level of Different Date Palm Cultivars to them in Qatar

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ABSTRACT

Leaf spot diseases are the most widely spread foliar disease in date palm. No records on the causal agent of these disease in Qatar. In this study pathogens were isolated purified and identified based on their morphological characters. Three genera of fungi (*Alternaria, Thielaviopsis* and *Helminthosporium*) were identified and confirmed to cause leaf spot disease in date palm in Qatar. The resistance of five date palm cultivars (khalas, Shish, Barhi, khnizy and Zirar) to these fungi was evaluated based on pathogenicity tests on palm under greenhouse conditions. Fifteen days post inoculation, the whole inoculated leaf showed typical symptoms like the origin from which it was isolated. *Alternaria* was the most aggressive one on all cultivars. All palms from five cultivars showed differences in resistance level between cultivars. Khenizi cv. was the most tolerant while Zarir cv and Shish cv was the most susceptible ones. On the other hand, Khalas cv showed different levels of resistance depend on the pathogen. The morphological identification was confirmed by molecular sequencing of ITS regions of each isolate. The length of ITS-rDNA sequence was 560 bp. Sequences were compared with those available in public gene bank databases and confirmed the presence of three genera *Alternaria alternate* had 92 and 95% sequence identity with *Alternaria alternate* (Kp174670.1 and MG722800.1 respectively), *A tenuissima* had 91% sequence identity with *Alternaria tenuissima* (KU93731501) *T. paradoxa* had 94% sequence identity with *Thieloviobsis paradoxa* (LC228644.1) and *H. velutinum* had 98% sequence identity with *Helminthosporium velutinum* (KY98435801)**.**

KEYWORDS: Date palm; *Phoenix dactylifera;* Leaf spot diseases; Pathogen resistance

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INTRODUCTION

Date palm in the Gulf region is considered as one of the most important agricultural crop due to its heritage and economical value. The government in Qatar is considering it as one of the main corps for food security.

Fungal pathogens attack different parts of the date palm which cause significant reduction in yield quantity and quality (El-Hassami *et al*., 2007; Abass *et al*., 2013). The dominant fungi associated with date palm are: *Thielaviopsis paradoxa* (Black scorch), *Helminthosporium spp.* and *Alternaria spp.* (leaf spots) and *Botrydiplodia theobromae* (Basal rot), (Al-Sharidy and Molan, 2008). Leaf spot diseases are widely spread in date palms orchard all over the world. More than 10 casual agents were recorded to give a raise to the symptoms of these diseases (Samir *et al.,* 2010). The development of symptoms mostly started as small, irregular to oval brown to black spots about 2-5 mm in length scattered on both upper and lower surface of the leaves. Sometimes, some spots expanded to form a blighted area concentrated on the lower part of rachis. Determining causal agents of fungal diseases is very crucial in applying the right measurements for plant protection. This can be achieved through identification of the disease symptoms, morphological identification of the pathogen then confirming this identity by molecular means.

Sensitivity to fungal pathogens differs between cultivars. This is mostly based on their genetic background. Both horizontal and vertical resistance play a role in controlling the spread of the disease in the plant. While many genes controlling the horizontal resistance (Vanderplank, 1963) vertical resistance is controlled by a single gene (Robinson, 1976). Screening cultivar for

resistance helps in reducing the spread of the diseases by planting cultivars with different resistance background specially for the vegetative propagated plants like date palm. Monoculture in other hand can results in a devastating consequence as it was the case of the spread of Bayoud disease in north Africa in Deglet Noor cultivar (El Hadrami *et al.,* 1997).

This paper reporting the casual agents for leaf spot diseases in Qatar and the tolerance of different cultivars to them.

MATERIAL AND METHODS

Isolation of the pathogens:

Samples of leaflets and fronds with visible leaf spot symptoms were collected from cultivar Khalas grown at the Agricultural Research farm, Rawdat Al Faras, Qatar. They were washed with tap water, surface sterilized with 2% sodium hypochlorite solution for 3 min, rinsed with sterile distilled water and allowed to dry. They were cut to about 1 cm^2 then plated on potato dextrose agar media (PDA) supplemented with chloramphenicol (Eddleman, 1998). Plates were incubated in the dark at 26±2 Cº for five days. Fungi isolates were purified using the single spore isolation technique (Constautinescu, 1988) then were stored at -20 Cº for further studies.

Pathogenicity test on detached leaflets:

Pathogenicity of fungal isolates were evaluated on visibly healthy detached leaflets. from Shish cultivar. Leaflets were surface disinfected by 1% sodium hypochlorite for two minutes, rinsed with distilled sterile water then allowed to dry on sterile filter paper. They were placed on moistened sterile filter papers inside sterilized plastic boxes. Spore suspensions (about 2.5 x 10^5 spores /ml) form each fungal isolate were prepared from 7 days old culture. They were sprayed on the detached leaflets using hand sprayer then incubated at 26 ± 2 °C for 14 days. Spraying with distilled water was used as a negative control. The study was conducted in three

replications. The re-isolation of pathogens from the inoculated leaves was conducted on PDA plates to fulfil Koch's postulates.

Morphological characterization.

The. The morphological identification was performed according to (Matsushima 1975). Hypha and conidia of 7 days old colonies grown on PDA plates were examined were examined using an optic light microscopic. Taxonomic keys and references were used to identify the fungi to the genus level (taxonomic systems of Ellis (1971), Nelson *et al*., (1983) and Paulin-Mahady *et. al.,* 2002)). Isolates were deposited in the culture collection of Qatar National Gene bank, Doha, Qatar.

Molecular identification

DNA was extracted from 50 mg of fungal biomass obtained from pure isolate growing on liquid PDA media using Qiagen Kit (Cat. No. 69106) Concentration was adjusted at 50 ng/µl using TE buffer pH 8.0. PCR reaction was performed using universal primers ITS1 (5': TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5': TCC TCC GCT TAT TGA TAT GC-3') (White *et al.,* 1990). The Polymerase Chain reaction (PCR) was carried out according to El-Badawy *et al.*, 2016. The homology of the resulting sequences was analyzed using BLAST (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/blast/).

Evaluation of resistance of date palm cultivars to the isolated pathogens:

Susceptibility test was conducted using 3-year-old trees from five date palm cultivars (khalas, Shish, Barhi, khnizy and Zirar) to evaluate their resistance to leaf spot diseases. by applying artificial inoculation with *Alternaria sp., Thielaviopsis. paradoxa and Helminthosporium sp.* under greenhouse conditions. Pathogen isolates spore suspensions were prepared from 7 days old culture. The spore concentration was adjusted to 2.5 x 10^5 spores /ml by using a hemocytometer. Pathogen spore suspension (100 ml/palm) was sprayed and the palms were covered for 72 hrs. Control plants were sprayed with sterile distilled water only. Each treatment was performed in triplicate. The pots were watered once a week. Symptoms was

recorded 14 days after inoculation. The disease development was rated using a 1-12 scale (Horsfall and Baratt 1945).

RESULTS

Fungal isolation:

Isolation and purification from naturally infected date palm leaves yielded 101 isolates of fungi. They were obtained from leaves exhibited leaf spots symptoms varied in shape and size (Fig 1).

Fig: 1. Naturally infected leaves and fronds of date palm with leaf spot symptoms.

Artificial inoculation with spore suspension on Shish cv gave typical symptoms of leaf spot disease (Fig 2). The re-isolation from the artificially infected leaves confirmed that all 101 isolates were pathogenic fungi.

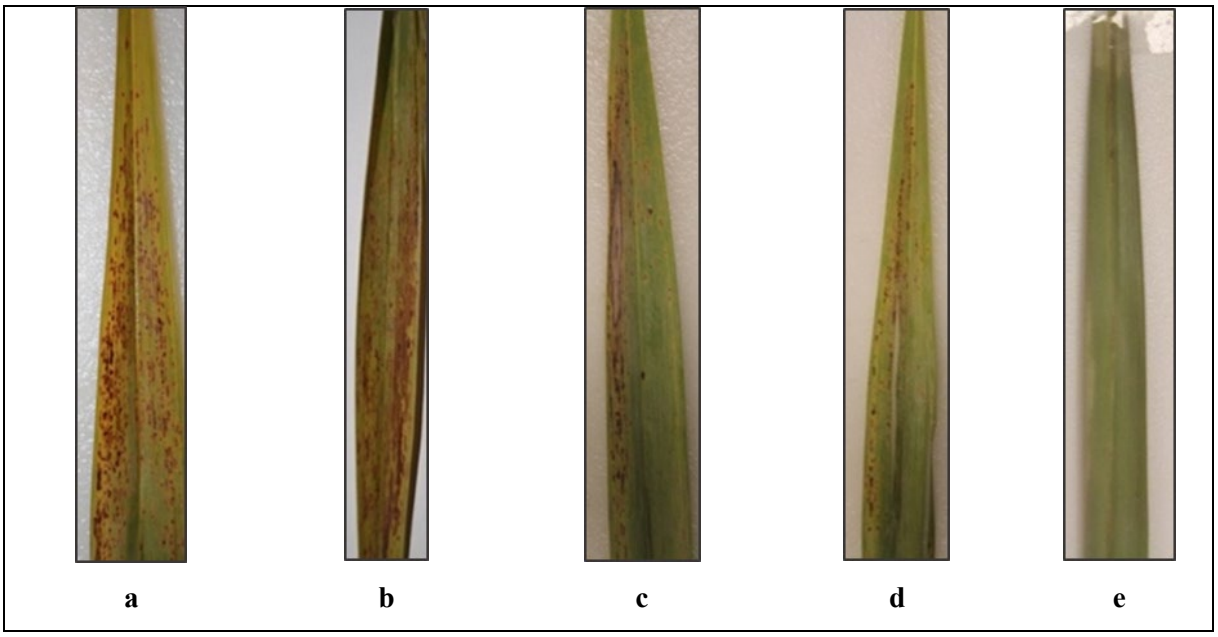

Fig: 2. Symptoms on plants of date palm inoculated with *Alternaria alternate* (a), *Alternaria tenuissima* (b), *Helminthosporium sp.* (c), *Thieloviobsis paradoxa* (d) and control plants (e).

Morphological identification:

The 101 isolates were classified to 3 genera based on their morphological characters on PDA plates. Theses genera were *Alternaria* (48 isolates), *Helminthosporium* (15 isoltes), *Thielaviopsis* (22 isolates) and 16 not identified isolates. *Alternaria* isolates grew rapidly and produced colonies with different shapes. The colony was flat, and was covered with grayish, short, aerial hypha. Its surface was grayish white at the beginning which later darkens and becomes greenish black or olive brown with light border. The reverse side was typically brown to black due to pigment production (Fig. 3. a & b). Hypha septate were brown. Conidia and proconidia appeared brown with an elongated beak like apical cell, often in chains and sometimes solitary. Conidia were large as shown in (Fig 3 e & f). *Helminthosporium* colonies were dark brown (Fig.3. c). Mycelium was immersed, composed of branched septate thick-walled hyphae. *Conidiophore*s were mostly unbranched. Conidia were single, pale brown to dark brown, straight or curved, round at the base and pointed at the other end (Fig.3 g). *Thielaviopsis* produced olive green color colony (Fig.3. d). It had creeping, almost hyaline hyphae which bore

two spore forms (micro- and macro- conidia). Microconidia were small circular shape. Macro-conidia were extruded in chains from the tips of short brown, thick- walled lateral hyphae (Fig.3. h). Morphologically three species could be determined. These were *Alternaria alternate, Alternaria tenuissima, Thieloviobsis paradoxa*. Species form *Helminthosporium* could not be determined from the morphological features.

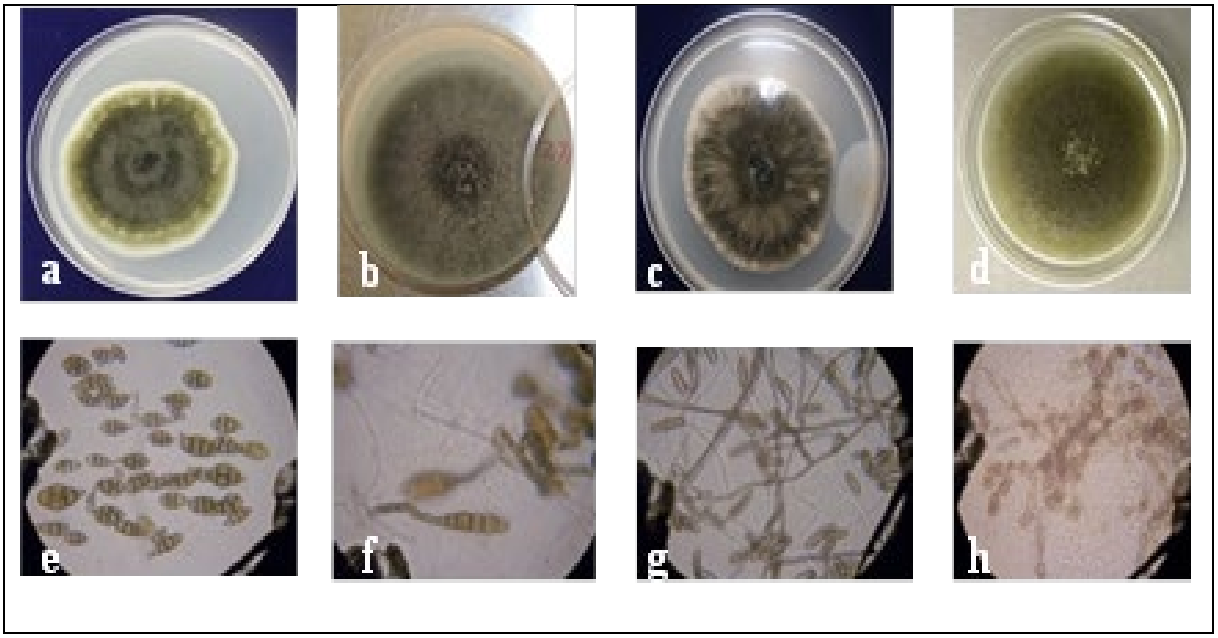

Fig.3. 10 days growing culture of pathogenic fungi.

1. On PDA plates: (a) *Alternaria alternate,* (b), *Alternaria tenuissima,* (c) *Helminthosporium sp.* and (d), *Thieloviobsis paradoxa*.
2. Microscopic features (e) *Alternaria alternate,* (f) *Alternaria tenuissima,* (g) *Helminthosporium sp.* and (h). *Thieloviobsis paradoxa.*

Molecular identification:

Genomic DNA of was extracted from 33 isolates fungal isolates representing the three genera. PCR using ITS1 and ITS4 primers produced approximately 560 bp. DNA**.** Sequences were compared with those available in public gene bank databases and confirmed the presence of three genera *Alternaria alternate,* accession number MG817475 and MG817477, (16 isolates), had 95 and 92% sequence identity with *Alternaria alternate* (MG722800.1 and Kp174670.1 respectively), *A tenuissima* accession number MG818910 (4 isolates), had 91% sequence identity with *Alternaria tenuissima* (KU93731501) *T. paradoxa,* accession number MG817480, (9

isolates), had 94% sequence identity with *Thieloviobsis paradoxa* (LC228644.1) *and H. velutinum,* accession number MG817476, (1 isolate), had 98% sequence identity with *Helminthosporium velutinum* (KY98435801)**.**

Resistance of date palm cultivars to the isolated pathogens:

The results of susceptibility test proved the ability of all tested isolates to induce spot symptoms on all tested cultivars after artificial inoculation. The typical symptoms of leaf spot were developed after 14 days in all treated palms with inoculum whereas the negative control remained symptomless during the same period. Cultivars showed different level of resistance to the pathogens. Khenizy cv showed the highest level of resistance while Shish and Zirar cultivar was the most susceptible ones among tested cultivars under all pathogens. (Table 1). Khalas is the only cultivar that showed significantly different reaction with each pathogen. Other four cultivars showed no significant differences in susceptibility of the same cultivar inoculated with different pathogens. *Alternaria alternata Helminthosporium velutinum and*, *Thielaviopsis paradoxa* had the same level of severity on Shish, Zarir and khenizy. *A alternata* was more aggressive on khalas and Barhi cultivars compared with the other three.

Table 1: Disease severity of leaf spot causal agents on five date palm cultivars

Cultivars	**Leaf spot pathogens**			**LSD**
	A. alternata	***H. velutinum***	***T. paradoxa***	
Khalas	5.67 ^{b}	4.00 ^{a}	2.00 ^{a}	1.332
Shish	8.00 $^{a\,A}$	6.67 $^{b\,A}$	6.67 $^{b\,A}$	1.883
Barhi	6.67 ^{b}	5.33 $^{a\,A}$	4.67 $^{a\,A}$	1.153
Zarir	8.33 $^{a\,A}$	7.00 $^{b\,A}$	7.67 $^{b\,A}$	2.492
Khenizy	3.00 ^{A}	2.33 ^{A}	2.67 ^{A}	1.49
LSD	1.627	1.151	1.879	

*Average indicated by the same small letter in each column has no significant differences at (P≤0.05%).
*Average indicated by the same capital letter in each row has no significant differences at (P≤0.05%).

DISCUSSION

Most pathogens attaching date palm are fungi (Zaid *et al*, 2002). Leaf spot diseases are widespread worldwide. High incidence of such diseases can be expected in the absence of control measures and the climate might change. These diseases may represent a real problem for date palm cultivation in the future. In this investigation, the main fungal species isolated were identified as *A. alternate, A. tenuissima*, *T. paradoxa* and *H. velutinum* based on morphological and molecular analysis. This is the first record on the causal agents of leaf spot diseases on date palm in Qatar. These pathogens except *A. tenuissima* were among the causal agents reported on similar climate conditions such as Saudi Arabia (Al-Sharidy and Molan, 2008), Kuwait (Mubarak *et al.,* 1994), Sultanate of Oman (Anonymous, 1993) and Iraq (Sarhan, 2001). They were also confirmed through morphological, molecular and phylogenetic studies (Eman and Kamal, 2011) and (Ammar and El-Nagar, 2011). The confirmation of morphological characterization by ITS sequences indicated that the morphological characteristics, based on the shape of the conidia on the mycelium are reliable feature of fungal identification. In this study, *A. alternate, A. tenuissima*, *T. paradoxa* and *H. velutinum* were identified to species level. *Alternaria sp.* and *Thieloviobsis paradoxa.* were the most predominant species isolated from visibly infected palms in this study. The same findings were reported by and Suleman *et al*., 2002 and Hernandez *et al.,* 2010.

A. tenuissima is known as a saprophytic fungus and opportunistic plant pathogen as mentioned by Chełkowski, and Visconti, 1992. Recently The species *A. tenuissima* has been reported to be the primary causal agent of Alternaria leaf spot and fruit rot in blueberry worldwide (Cline 1996; Greco *et al.,* 2012 and You *et al*., 2013). In this study, it was confirmed to be one of the causal agents of leaf spot diseases on the date palms. Its pathogenicity was observed on the detached leaves within 14 days after inoculation and was confirmed by Koch's postulates. This can show the potentiality of this fungi to attach different part of the palm.

The results of susceptibility test showed different resistance level in the interaction between cultivars and pathogens. It is clear that genetic background of the cultivars played a role in controlling the pathogen colonizing the tissue and its spread. It can be recommended to use

mixed cultivars in plantation instead of monoculture to reduce hazards of these diseases. This also will reduce the selection pressure on the pathogen and slow down it mutation to improve its pathogenicity. Khalas reactions to different pathogens showed that it has different genetic background than other four cultivars. It had three levels of resistance from high to medium to *T. paradoxa, H. velutinum* and. *Alternaria alternata* respectively. Zarir and Shish showed the most susceptible reactions. This has to be taken in consideration in designing programs of plant protection. They are however good candidates to be used as positive control in pathogenicity tests and in vivo culture of fungi isolates. This is the first evaluation of these cultivars in Qatar.

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