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Morphological and Molecular Characteristics of Alternaria citrus stem end rot

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Abstract

Citrus stem end rot symptoms on citrus fruit were observed in Alsuwaira orchard citrus since 2015 in Wasit province, Iraq. Infected citrus fruits were collected to isolate and characterize the pathogenic factors. The isolates showed that Alternaria spp. has been involved in infected citrus fruit. Genetic characteristics were analyzed based on sequence of rDNA-internal transcribed spacer (ITS) region. The phylogenetic tree based on rDNA- ITS analysis showed that the Alternaria alternata the main pathogenic factor of citrus stem end rot.Molecular identity of rDNA-ITS region of the present isolates were deposited in the GenBank database under the eight accession numbers from MK121457 to MK121464.

Keywords*: Alternaria alternata, Citrus stem end rot, fungal pathogens isolation and identification*

I. Introduction:

Citrus plants consider one of the main fruit trees in the world (Sidana *et al*., 2013). Iraq annually produce s about 72816 tons of citrus fruitsin three main regions Kerbala, Diyala and Al-Suwaira. These plants are susceptible to a large number of diseasescaused by plant pathogens from bloom to harvesting and post harvesting (Embaby *et al*., 2013). The common fungi of citrus fruits include *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp., *Curvuleria* sp., *Alternaria* sp., *Colletotrichum* sp. *Guignardia* sp. and *Diplodia* sp. (Khan *et al*., 2015). Four different diseases caused by *Alternaria* spp. have been registered on citrus trees: Alternaria leaf spot of rough lemon, Alternaria brown spot of tangerines, Alternaria black rot of several citrus fruitsand Mancha foliar of Mexican lime (Akimitsu *et al*., 2003). Rang *et al*., (2002) studied morphologically Alternaria isolates of black rotted Minneola Tangelo, Mandarin and Navel orangethat belong to species of *A. alternata*, *A. pellucida*, *A. citri*, *A. tenuissima* and *A. arborescens*.Those pathogens could cause a massive loss (30-50%) in different stages of some susceptible cultivars. Alternaria found on citrus fruits as stem end rot and internal core rot or black rot.The causal agent of citrus black rot was identified as *A. tenuissima* in Iran (Mojerlou and Safaie, 2012).*Alternaria alternata*is the most abundant fungal pathogen isolated from citrus fruit stem end rot (Embaby *et al*., 2013; Abdelmalek and Salaheldin, 2016; Galsurker *et al*., 2018). This study aimed to characterize fungal pathogens causal of citrus stem end rot using morphological and molecular identification. In addition, our further goal is to determine the pathogenicity and phylogenetic of the Alternaria isolates from citrus stem end rot in Iraq.

II. Materials and methods

Fungal isolate

Citrus fruit stem end rot samples were collected from citrus orchards in Al-Suwaira region, Wasit province south of Baghdad- Iraq in 2018. The sample surface sterilized with 1% sodium hypochlorite for 2 min. pieces were cutted of from citrus fruit stem end rot and then put on potato dextrose agar (PDA) medium and incubated at 25°C ± 2°C for 3 days. The fungi cultures were ~~and~~ identified as *Alternaria* spp. based on morphological characteristics as described by Navi *et al*., (1999) and maintained on a slant PDA at 4°C for next study.

Pathogenicity of *Alternaria* spp. isolates

To confirm pathogenicity of *Alternaria* spp. isolates, healthy citrus fruits were surface-sterilized in three replications.20 µl of conidial suspension containing 10^4 conidia/ml of *Alternaria* sp. was placed on fruit under the pedicels and put in sealed sterilized plastic bags and incubated at 25°C ± 1°C for 7 days. Citrus fruits were circle, regularly watered and monitored forthe disease development (Singh and Khanna, 1966).

Alternaria DNA extraction

The Alternaria pathogen was grown on PDB medium at 25°C for 7 days. Alternariamycelia were harvested and washed repeatedly with distilled sterilized water.Mycelium (1 g) was crushed in liquid nitrogen and then mixed well with 5 ml buffer [75mM Nacl,25mM EDTA(pH8) and 20mM Tris (pH7.5)].The solution was incubated with 100 ml lysozyme at 37 ºCfor 60 min.600 µl (10% SDS) and 140 µl (proteinase K)were added to the solution and incubated for 2 h at 55 ºC with gentle shaking.The 5 ml chloroform was added and mixed to 30 min at 25 ºC and centrifuged for 20 min at 4500 rpm. The DNA pellets were washed with 70% ethanol for twice and air dried.Next, the pellets were re-suspended in 100 µl sterile TE(10mMTris-Hcl buffer and 1mMEDTA-pH8) and stored at -20 ºC in freezer. Finally,Alternaria isolate DNAs were electrophoresedin 1% agarose gels (Kareem and Hassan, 2015).

PCR amplification

The ITS1 and IT4 primers were used for PCR amplification of the pathogen of citrus stem end rot. The PCR reaction contained 5 µl master mix,5 µl Alternaria isolates DNA and 2 µl each of the forward primer ITS1:TCCGTAGGTGAACCTGCGG,reverse primer ITS4:TCCTCCGCTTATTGATATGC. The PCR program: 1 cycle at 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 1 cycle of 72°C for 5 min. The PCR products were analyzed on 1% agarose gel with gel elution kit (white *et al*., 1990).

Genetic DNA sequencing and phylogenetic tree

The PCR products were sequenced by the dideoxy nucleotide chain termination method (Sanger *et al*., 1977). The DNA sequencing was checked using the BLAST program through NCBI (http://www.ncbi.nlm.nih.gov)website.After multiple alignments, phylogenetic analysis of Alternaria sequences

was achieved in MEGA7 software with Clustal W program (Kumar *et al*., 2016). The phylogenetic tree has been reconstructed using the neighbor joining method (Saitou and Nei, 1987) with boots trapped 500 replication

III. Results and Discussion

Fungal Isolate

The isolated fungi fromcitrus fruit stem end rot produced abundant, branched, septate, olive-brown mycelia. Conidiophores were septate andsimplewith terminal conidia, which were individual conidia or in short chainswith short conical beak or beakless. The conidial and mycelium characteristics from culture were similar to the conidia isolated from infected citrus fruit stem end rot. Based on the morphological characters (**Figures 1** and **2**), the fungus was identified as *Alternaria alternata*.

Figure 1. Alternaria citrus fruit stem end rot symptom on fieldcitrus fruits.

Figure 2. Conidial and short chainsof *Alternaria alternata* infected citrus fruit stem end.

Pathogenicity of *Alternaria* spp. isolates

The pathogen was confirmed as a pathogenic factor on Iraqi citrus fruits and conformed to disease characteristics as observed in the field symptoms of citrus fruit that were stem end rot. Later, these rot spots were initially look like light brown and afterward turned to dark brown (**Figure 3**).

Figure 3. Alternaria citrus fruit stem end rot symptom on citrus fruits in laboratory.

Alternaria sequencing of ITS Region

The results showed that the sequences of Alternaria isolates had the highest similarity of 99-100% with *A. alternata* species. Molecular identity of rDNA-ITS region of the present isolates were deposited in the GenBank database under the eight accession number MK121457-MK121464. The actual size of the PCR product was 550 bp. BLAST analysis of the 550bp amplicon showed 99% similarity with *A. alternata* sequences (MG744601) from Tunisia and 100% similarity with sequences (MG827243) and (MF029625)from Taiwan and South Korea respectively**(Table.1)**.

Table.1: Genetic similarity of Alternaria Fungi associated with the stem end rot of citrus fruits.

Alternaria isolates	% Genetic similarity										
MK121457-*Alternaria alternata*-Altalt1											
MK121458-*Alternaria alternata*-Altalt2	99										
MK121459- *Alternaria alternata*-Altalt3	100	100									
MK121460- *Alternaria alternata*-Altalt4	100	100	100								
MK121461- *Alternaria alternata*-Altalt5	100	100	100	100							
MK121462- *Alternaria alternata*-Altalt6	99	98	99	100	100						
MK121463- *Alternaria alternata*-Altalt7	100	100	100	100	100	100					
MK121464-*Alternaria alternata*-Altalt8	100	99	100	100	100	99	100				
MG827243.1- *Alternaria alternata*-Taiwan	100	99	100	100	100	100	100	100			

MF029625.1-*Alternaria alternata*–South Korea	99	98	100	100	100	99	100	99	100		
MG744601.1- *Alternaria alternata*-Tunisia	100	100	99	99	99	99	99	100	99	99	
KF312465.1- *Rhizoctonia solani*	79	76	77	79	78	79	78	78	79	80	78

The result shows *Alternaria alternata as* the main pathogen of citrus fruit stem end rotdisease in Iraqi citrus orchards (**Figure 4**).According to analysis above, eight isolates were associated to *A. alternate* and clustered with several *A. alternata* reference from other countries. The phylogenetic analysis of*A. alternata* isolates using all sequenced markers (ITS) made it possible to identify*A. alternata* as a main monophyletic clade. *Rhizoctonia solani*(KF312465) formed a distinct clade as an out croup member that was clearly separated from the other isolates of*A. alternata*in the neighbor-joining tree.

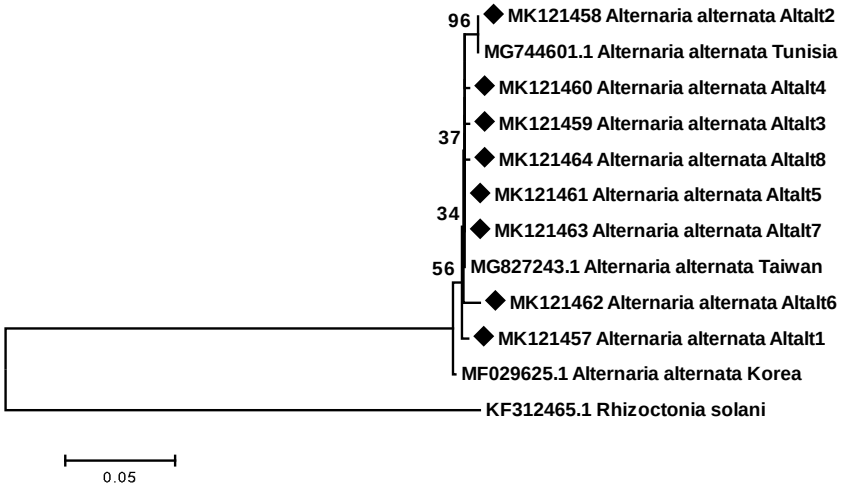

Figure 4. Phylogeny tree showing relationships among the related *Alternaria alternata* based on their ITS sequences. *Rhizoctonia solani*isolate KF312465 was used to root the phylogeny.

IV. DISCUSSION

This study showed that numbers of Alternaria isolates were associated with citrus fruit stem end rot causedsignificant loss of citrus fruits in Al- Suwaira orchards of Wasit, Iraq.The results of this study indicated that eight Alternaria isolates were involved in quality degradation citrus fruits of Al-Suwaira. Peever *et al*. (2005) described*Alternaria spp*. that caused several diseases of citrus trees, including *Alternaria* leaf spot, brown fruit spot and black rot. The stem end rot disease is an important disease of citrus fruit in warm and humid citrus growing regions (Fischer *et al*., 2009). *Alternaria alternata* caused Alternaria citrus fruit stem end rot was characterized for the first time in Iraq at molecular level using rDNA-ITS region. rDNA-ITS analysis indicated that Alternaria citrus fruit stem end rot resulted from *Alternaria alternata*. On the basis of the work by Garganese

et al., (2016), phylogenetic analyses were initially conducted by ITS region. It made it possible to cluster 8 isolates with *A. alternata* references. In particular, a monophyletic clade was obtained. Sharma *et al*. (2012) reported that the genomic regions of 17 *Alternaria* spp. showing 99% - 100% sequence identity with *Alternaria alternata*. Related morphological characterization, good results were observed when isolates of *Alternaria* produces conidiophores and associated conidial chains. This result agreed withGarganese*et al*., (2016) outcomes. All of the investigated isolates were capable to cause citrus fruit stem end rot.

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