

Morphological and Molecular Characteristics of *Alternaria* citrus stem end rot

¹T. A. Kareem, ²Dareen S. Jamel, ³Abdulzahra Jabar Ali, ⁴R. I. Saeed

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 fruits in three main regions Kerbala, Diyala and Al-Suwaira. These plants are susceptible to a large number of diseases caused 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 fruits and 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 orange that 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 $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ 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 $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 7 days. Citrus fruits were circle, regularly watered and monitored for the disease development (Singh and Khanna, 1966).

Alternaria DNA extraction

The *Alternaria* pathogen was grown on PDB medium at 25°C for 7 days. *Alternaria* mycelia 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°C for 60 min. 600 μl (10% SDS) and 140 μl (proteinase K) were added to the solution and incubated for 2 h at 55°C with gentle shaking. The 5 ml chloroform was added and mixed to 30 min at 25°C 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 (10mM Tris-HCl buffer and 1mM EDTA-pH8) and stored at -20°C in freezer. Finally, *Alternaria* isolate DNAs were electrophoresed in 1% agarose gels (Kareem and Hassan, 2015).

PCR amplification

The ITS1 and ITS4 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 500 bootstrap replication.

III. Results and Discussion

Fungal Isolate

The isolated fungi from citrus fruit stem end rot produced abundant, branched, septate, olive-brown mycelia. Conidiophores were septate and simple with terminal conidia, which were individual conidia or in short chains with 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 field citrus fruits.



Figure 2. Conidial and short chains of *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- <i>Alternaria alternata</i> -Altalt1										
MK121458- <i>Alternaria alternata</i> -Altalt2	99									
MK121459- <i>Alternaria alternata</i> -Altalt3	100	100								
MK121460- <i>Alternaria alternata</i> -Altalt4	100	100	100							
MK121461- <i>Alternaria alternata</i> -Altalt5	100	100	100	100						
MK121462- <i>Alternaria alternata</i> -Altalt6	99	98	99	100	100					
MK121463- <i>Alternaria alternata</i> -Altalt7	100	100	100	100	100	100				
MK121464- <i>Alternaria alternata</i> -Altalt8	100	99	100	100	100	99	100			
MG827243.1- <i>Alternaria alternata</i> -Taiwan	100	99	100	100	100	100	100	100		

MF029625.1- <i>Alternaria alternata</i> –South Korea	99	98	100	100	100	99	100	99	100		
MG744601.1- <i>Alternaria alternata</i> -Tunisia	100	100	99	99	99	99	99	100	99	99	
KF312465.1- <i>Rhizoctonia solani</i>	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 rot disease in Iraqi citrus orchards (**Figure 4**). According to analysis above, eight isolates were associated to *A. alternata* 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 group member that was clearly separated from the other isolates of *A. alternata* in the neighbor-joining tree.

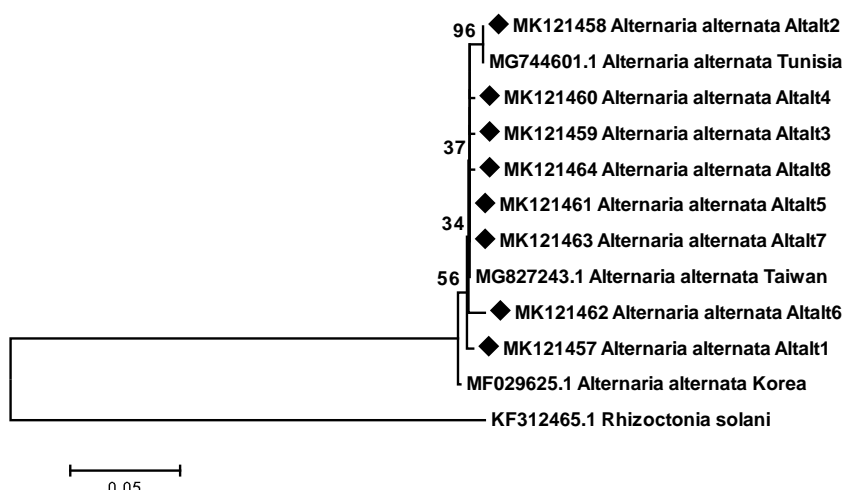


Figure 4. Phylogeny tree showing relationships among the related *Alternaria alternata* based on their ITS sequences. *Rhizoctonia solani* isolate KF312465 was used to root the phylogeny.

IV. DISCUSSION

This study showed that numbers of *Alternaria* isolates were associated with citrus fruit stem end rot caused significant 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 with Garganese *et al.*, (2016) outcomes. All of the investigated isolates were capable to cause citrus fruit stem end rot.

References

1. Abdelmalek G.A.M. and T.A. Salaheldin. 2016. Silver Nanoparticles as a Potent Fungicide for Citrus Phytopathogenic Fungi. J Nanomed Res 3(5): 00065. DOI: 10.15406/jnmr.2016.03.00065.
2. Akimitsu K., T.L. Peever and L.W. Timmer. 2003. Molecular, ecological and evolutionary approaches to understanding *Alternaria* diseases of citrus. Molecular Plant Pathology. 4(6): 435–446.
3. Embaby E.M., M. Hazaa, F. Laila Hagag, I. Talaat El-Sayed and F.S. Abdel- Azem. 2013. Decay of some citrus fruit quality caused by fungi and their Control: II- Control *Alternaria* rot or core rot decay by using some alternative fungicides. J Appl Sci Res. 9(11): 5671-5678.
4. Fischer I.H., M.O. Ferreir, M.B. Spósito and L.Amorim. 2009. Citrus postharvest diseases and injuries related to impact on packing lines. *Scientia Agricola*. 66(2): 210-217.
5. Galsurker O., S. Diskin, D. Maurer, O. Feygenberg and N. Alkan. 2018. Fruit Stem-End Rot. *Horticulturae*. (4)50;doi:10.3390/horticulturae4040050.
6. Garganese F., L. Schena, I. Siciliano, M. I. Prigigallo, D. Spadaro, A. De Grassi and *et al.* 2016. Characterization of Citrus-Associated *Alternaria* Species in Mediterranean Areas. PLoS ONE 11(9):e0163255. doi:10.1371/journal.pone.
7. Kareem, T.A. and M.S.Hassan. 2015. comparison of *Rhizoctonia solani* isolated from soil in Baghdad, Iraq genetically with world isolates. *Donnish journal of Agricultural research*. 2(3):029-035.
8. Khan A., S. Iram and A. Rasool. 2015. Pathogens identification and characterization that compromised citrus fruit quality in selected orchards of Sargodha. *Int. J. Environ. Sci. Toxic. Res*. 3(4):54-59.
9. Kumar, S., G. Stecher and K. Tamura. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* 33(7):1870-1874.
10. Mojerlou S. and N. Safaie. 2012. Phylogenetic Analysis of *Alternaria* species Associated with Citrus Black Rot in Iran. *J. Plant Pathol. Microb.* 3:144. doi:10.4172/2157-7471.1000144.
11. Peever T.L., L. Carpenter-Boggs, L.W. Timmer, L.M. Carris and A. Bhatia. 2005. Citrus Black Rot is caused by Phylogenetically Distinct Lineages of *Alternaria alternata*. *The American Phytopathological Society*. 95(5).

12. Rang J.C., P.W. Crous, G.R.A. Mchau, M. Serdani and S.M. Song. 2002. Phylogenetic analysis of *Alternaria* spp. associated with apple core rot and citrus black rot in South Africa. *Mycol Res* 106: 1151-1162.
13. Saitou N. and M. Nei. 1987. The neighbor joining method: A new method for reconstructing phylogenetic tree. *Mol. Biol. Evol.* 4(4): 406-425.
14. Sanger, F. Ai., G.M. Barrell, B.G. Brown, N.L. Coulson, A.R. Fiddes, C.A. Hutchison, P.M. Slocombe, and M. Smith. 1977. Nucleotide sequence of bacteriophage phi x 174DNA. *Nature*. 24: 265(5596): 687-695.
15. Sharma M., R. Ghosh, U.N. Mangala, K.B. Saxena and S. Pande. 2012. *Alternaria tenuissima* Causing Alternaria Blight on Pigeonpea [*Cajanus cajan* (L.) Millsp.] in India. *Plant Disease*, 96, 907 .
16. Singh, R. S. and R. N. Khanna. 1966. Black rot of mandarin orange caused by *Alternaria tenuis* Auct. *Plant Dis. Rep.* 50:127-131.
17. White, T.J., T. Bruns, S. Lee, and J. Talyor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis ,M.,A., Gelfand , D.H., Suinisky , J.J. and White ,T.J. Eds., *PCR protocols: a guide to methods and applications*, Academic press, San Diego, 315-322.