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Molecular Diagnosis of *Alternaria* spp. Causing Citrus Dieback in Central Iraq

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Abstract
Dieback diseases of hardwoods are a general term used to refer to a multitude of diseases primarily caused by fungal pathogens.This study was conducted to identify the genetic polymorphisms of three local species of *Alternaria* that isolated from infected citrus leaves,fruits,branches stains from Karbala city. One genetic locus covering a portion of the ribosomal subunit of the RNA gene was amplified in this study. A direct sequencing strategy was performed for the observed PCR amplicons in the amplified ribosomal locus. Subsequently, a comprehensive phylogenetic tree was constructed in the observed variants to assess the accurate phylogenetic distances with other relative fungal sequences. Our results indicated the presence of entire homology between our investigated samples and several species within *Alternaria*, namely *A. alternata*, *A. destruens*, and *A. tenuissima*, while *A. brassicicola* was slightly tilted from the other observed variants in the comprehensive tree. Interestingly, all observed variations were taken one pattern of phylogenetic distribution, represented by the close positioning in the vicinity to *A. alternata* species. However, the present tree showed that there was extremely close homology between the incorporated species of *A. alternata*, *A. destruens*, and *A. tenuissima*. Furthermore, the phylogenetic tree provided further tools regarding the guaranteed identity of the investigated samples. Therefore, the utilization of PCR-sequencing strategy in three analyzed DNA samples of these local fungal species have presented a confirmed identity of these strains within the *Alternaria* sequences.

Keywords: PCR, *Alternaria* species,Pathoginicity.

Introduction

Fungi are a vast kingdom of organisms with a range between 1.5 and 6 million species (Hawksworth et al. 2017), but only a modest part, around 140.000 species, is phenotypical and genetically described (Hibbett et al. 2016). Usually, fungi have been identified by morphology on pure cultures in agar medium. The main problem is that many species are difficult to isolate and culture, and even to classify (Ihrmark et al. 2012 . Fungs share a common set of symptoms, i.e., vascular necrosis (canker) of phloem and xylem tissues along with terminal branch and scaffold limb dieback. Additional symptoms can be associated with these diseases; however, this is depended upon the biology of individualr dieback pathosystem and the response of the host to this disease (Blanchette and Biggs, 2013) According to phylogenetic studies and sporulation patterns, the members of Alternaria genus have been clustered in several species-groups (Hong et al. 2005; Simmons 2007). The majority of them comprise of either saprophytic or plant pathogenic species of filamentous fungi, responsible for a number of crop diseases. The pathogenic members of this genus can cause losses in agriculture by reducing crop yield and through spoilage during storage (Ostry 2008).). *Alternaria* damage to young vegetative shoots varies from small circular leaf spots to large necrotic blighted lesions that cover a major portion of the leaf. Lesions tend to extend out along the veins. Damage to vegetative growth often results in

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severe leaf drop. Twig infection and the defoliation that commonly follows infection of the leaf blade, causes die-back of the shoot apices. Infected twigs provide the most continuous source of infection of lower hanging fruit.

The polymerase chain reaction-based assays have long been used as tool for fungal detection (Ferrer et al. 2001). PCR-based methods for rapid and specific detection of *Alternaria solani* (Kumar et al. 2013), *Alternaria brassicicola*, *Alternaria alternate*, *Alternaria helianthi* and *Alternaria dauci* (have been reported. Further development of a rapid, reliable and specific detection of the pathogen using PCR-based diagnostic markers of small size and ease of amplification is quite useful for implementing remedial measures against the pathogen. Initially, internal transcribed spacers (ITS) had been the most preferred target for primer designing due to limited fungal genome data (Martin and Rygiewicz 2005).

Materials & Methods

Alternata spp. species

Alternata spp. was isolated from infected citrus leaves,fruits ,brunches stains from Karbala city, which were surface sterilized by immersing them in a 1% sodium hypochlorite solution for three minutes, then washed with sterile distilled water and dried by placing them between two stereilized filter papers and transferred to petri dishes containing Potato Dextros Agar containing an antibiotic medium. Chloramphenicol 10 mg / L, then the plates were incubated with the incubator at 25°C for a period of five days (Aneja, 2004), and the isolated fungus was diagnosed based on the classification key prepared by (Ellis 1971).

The severity of infection

After two months of treatment, the percentage of infection and its severity were calculated on the leaves according to the Vakalunaki (1990) method and as follows:

Table (1): calculated the severity of infection on the leafs

Stage	Pathological evidence
0	**Without spots**
1	**Spots less than 25% of surfase area**
2	**Spots 26-50% of surfase area**
3	**Spots 51-75% of surfase area**
4	**Spots 76-100% of surfase area**

The severity of infection= leave number of stage 1* Pathological evidence+ leave number of stage 2* Pathological evidence + ……/ total leave number* higher stage

Pathogenicity test

Different species of *Alternaria* were obtained from fruits affected by “brown spot” from mandarin trees “clementina” , orange “local”, located in (Karbala, Iraq). For this study, the species were selected because they showed low and high degrees of aggressiveness, respectively, in unwounded fruits .The species *A. alternate*, *A. tenuissima* ,*A. destruens* were grown in PDA medium at 25◦C to serve as inoculum in the different studies. Fungal

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pathogenicity was evaluated by measuring the growth of the lesion produced after inoculation by two ways spray and injection of unwounded fruits with each one of the isolates. Prior to inoculation, fruits were disinfected with a solution of NaClO (0.5%) for 5 min and then washed with sterile water and dried with cellulose paper previously autoclaved. Ten similar fruits were used in each of the inoculation assays described below.The inoculations were performed under sterile conditions in a laminar flow cabinet. After inoculation, they were kept in a chamber at 20◦C and 85% relative humidity. At 7 days post-inoculation, the growth of the fungus was determined by measuring the diameter of lesion produced in mm.

DNA extraction

The genomic DNA of the fungal samples was extracted using Genaid Kit according to the manufacturer's instructions (Geneaid Biotech, Taiwan). The concentration and purity of DNA were measured by a nanodrop (BioDrop μLITE, BioDrop co., UK), while the DNA integrity was checked by a standard 0.8% (w/v) agarose gel electrophoresis that is pre-stained with a higher concentration of ethidium bromide (0.7 μg/ml) in TAE (40 mM Tris-acetate; 2 mM EDTA, pH 8.3) buffer, using a 1 kb ladder as a molecular weight marker (Cat # D-1040, Bioneer, Daejeon, South Korea). The isolated DNA was used as a template for PCR.

PCR

One PCR fragment was selected for amplification, which was designed to cover one ribosomal locus within the fungal genomic sequences. The lyophilized primers were purchased from Bioneer (Bioneer, Daejeon, South Korea). The PCR reaction was performed using *AccuPower* PCR premix (Cat # K-2012, Bioneer, Daejeon, South Korea). Each 20μl of PCR premix was contained 1 U of Top DNA polymerase, 250 μM of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, 1.5 mM of $MgCl_2$. The reaction mixture was completed with 10 pmol of each primer and 50 ng of genomic DNA. The following program was applied in PCR thermocycler (MyGenieTM 96/384 Thermal Block, Bioneer, Daejeon, South Korea). The amplification was begun by initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94˚C, annealing (61 ˚C for 1 min), and elongation at 72˚C, and was finalized with a final extension at 72°C for 10 min. Amplification was verified by electrophoresis on an ethidium bromide (0.5 mg/ml) pre-stained 1.5% (w/v) agarose gel in 1× TBE buffer (2 mM of EDTA, 90 mM of Tris-Borate, pH 8.3), using a 100-bp ladder (Cat # D-1010, Bioneer, Daejeon, South Korea) as a molecular weight marker. It was made sure that all PCR resolved bands are specific and consisted of only one clean and sharp band in order to be submitted into sequencing successfully.

DNA Sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from termini, forward, and reverse, according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed nucleic acid sequences of local samples with the retrieved reference sequences of the fungal database, the virtual positions, and other details of the retrieved PCR fragments were identified.

Interpretation of sequencing data

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The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

Comprehensive phylogenetic tree construction

A specific comprehensive tree was constructed in this study according to the neighbor-joining protocol described by Sarhan *et al.* (2020). The observed variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server (Zhang *et al*. 2000). Then, a full inclusive tree, including the observed variant, was built by the neighbor-joining method and visualized as an unrooted tree using iTOL suit (Letunic and Bork, 2019). The sequences of each classified phylogenetic species-group in the comprehensive tree were colored appropriately.

Results

Diagnosis

The pathogen was diagnosed by using the morphological features of the three species, as well as by using light microscopy and as shown in figure (1).

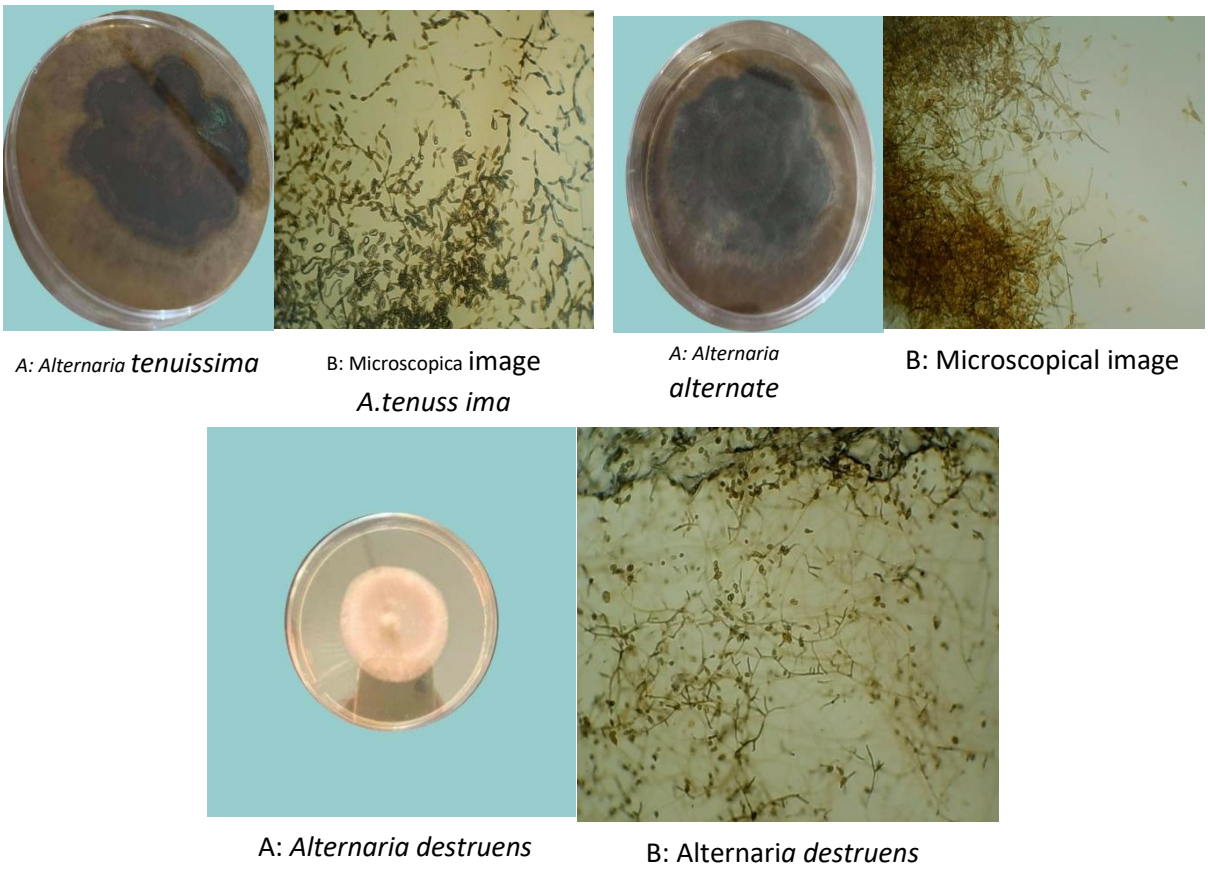

Figure (1): morphological and microscopical features

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The severity of infection

The severity of infection differed between the plants treated with fungi, as the results showed that the leaves of the Mandarin plant showed the highest rate of infection severity when they were treated with *Alternaria destruens*, reaching 75%. Whereas, the lowest percentage of infection severity was in orange leaves when *Alternaria alternate* was treated, and it reached 8%, as shown in figure (2).

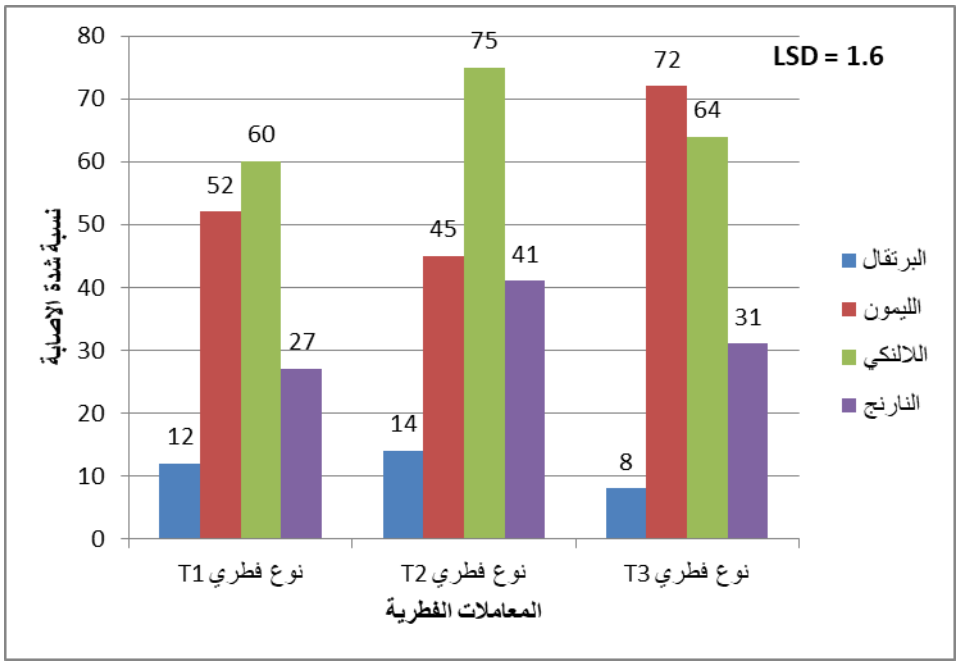

Figure (2): The severity of infection.

Spot area rate

The results of the current study showed the different areas of the spot on orange fruits according to the fungal treatments. It was found that the treatment *Alternaria alternate* caused a residual area if it reached 4.67 mm, while the treatment with *Alternaria destruens* showed the lowest spot diameter if it reached 2.83 mm, by using spray method as shown in figure (3).

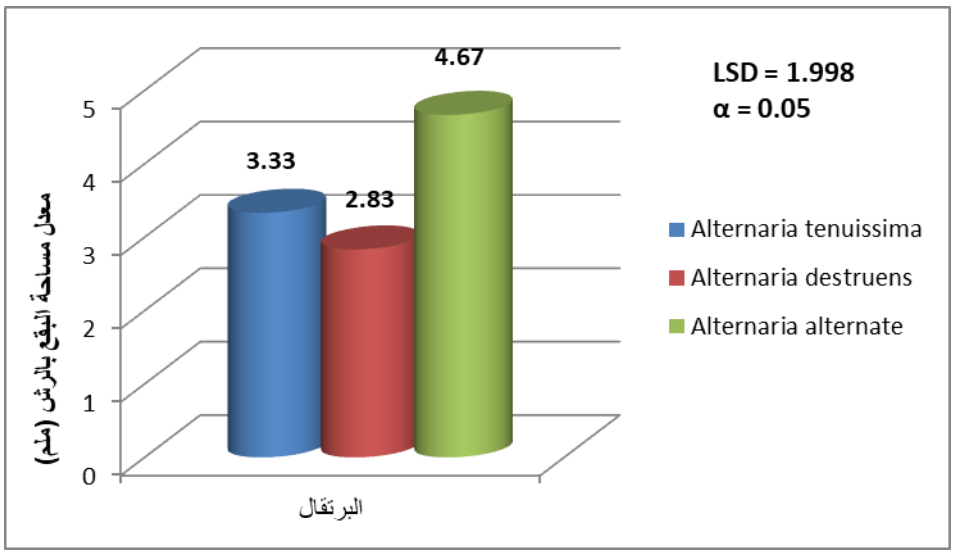

Figure (3): Spot area rate on orange by using spray method

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While, The results of the current study showed the different areas of spot on orange according to the fungal treatments. It was found that the treatment *Alternaria destruens* caused a residual area if it reached 4.2 mm, while the treatment with *Alternaria alternate* showed the lowest spot diameter if it reached 2.33 mm, by using injection method as shown in figure (4).

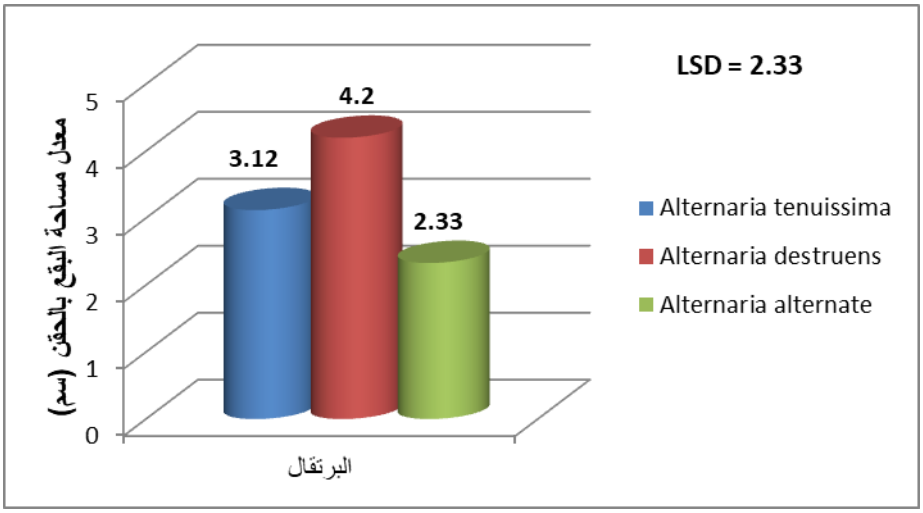

Figure (4): Spot area rate on orange by using injection method

Also, the findings showed the different areas of the spot on Mandarin according to the fungal treatments. It was found that the treatment *Alternaria alternate* caused a residual area if it reached 10.83 mm, while the treatment with *Alternaria* ***tenuissima*** showed the lowest spot diameter if it reached 4 mm, by using spray method as shown in figure (5).

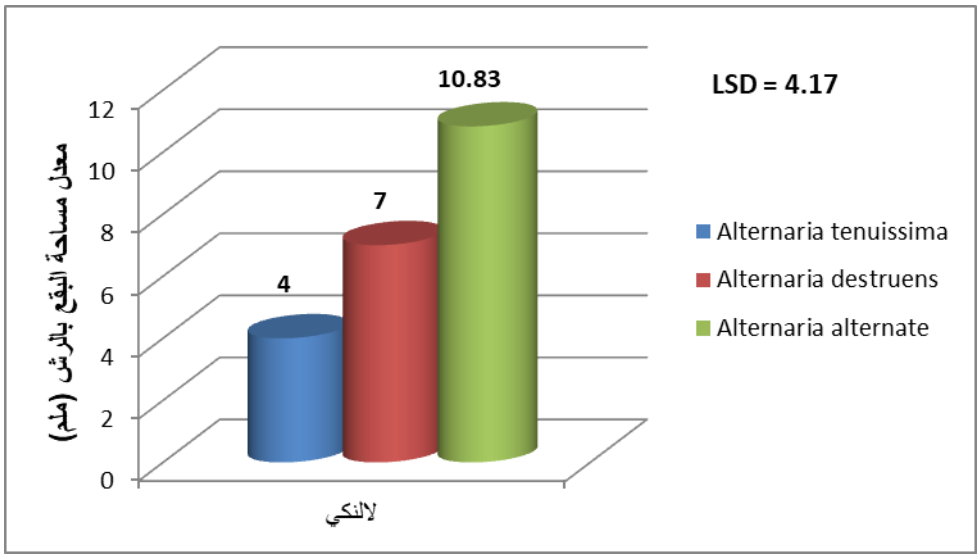

Figure (5): Spot area rate on Mandarin by using spray method

While, the findings showed the different areas of the spot on Mandarin according to the fungal treatments. It was found that the treatment *Alternaria alternate* caused a residual area if it reached 9.7 mm, while the treatment with *Alternaria* ***tenuissima*** showed the lowest spot diameter if it reached 2.5 mm, by using injection method as shown in figure (6).

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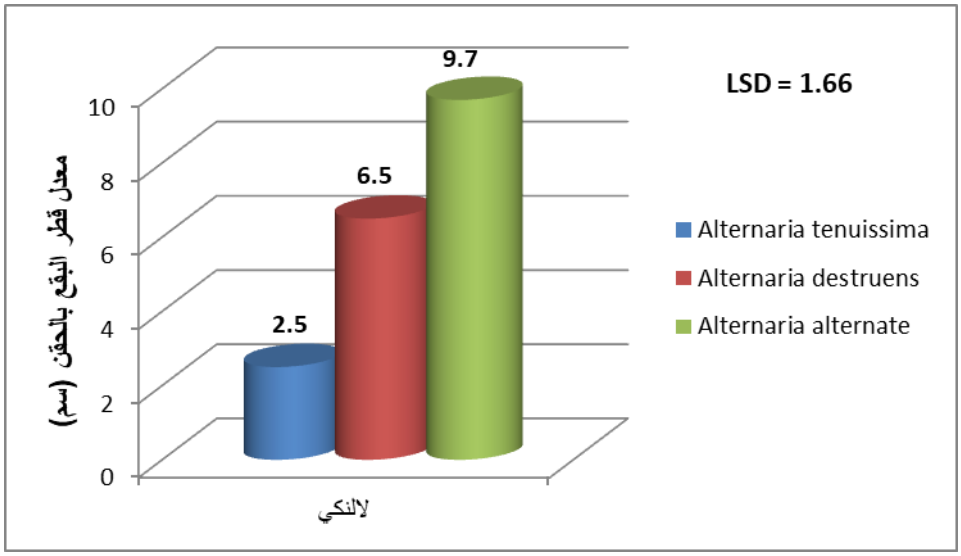

Figure (6): Spot area rate on Mandarin by using spray method

On the other hand, when comparing the average diameter of spots in the fruits of both oranges and Mandarin when treating with fungi, it was found that the average diameter of spots in oranges was much higher than the average diameter of spots in Mandarin , by using injection method as shown in figure (7).

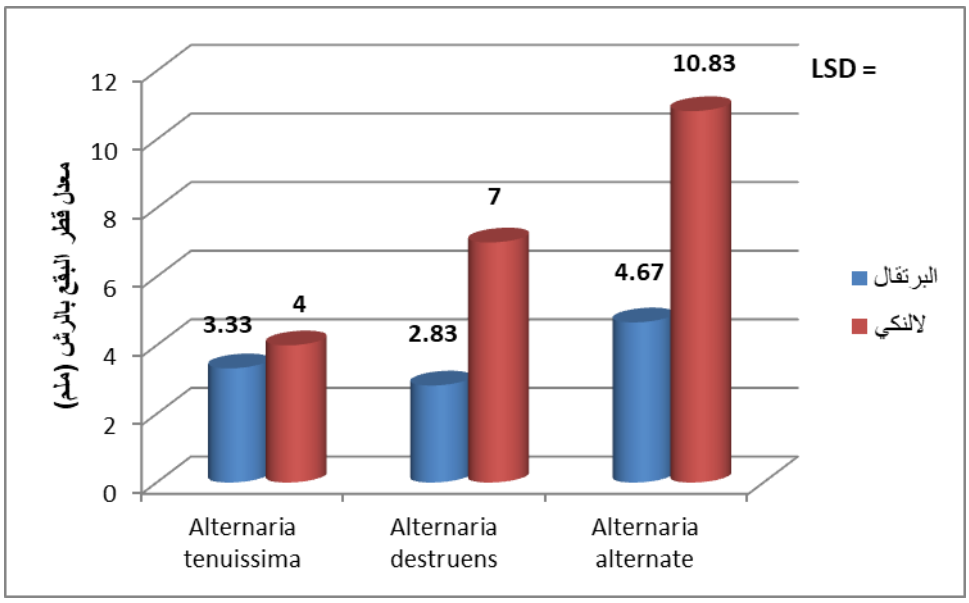

Figure (7): Spot area rate between orange and Mandarin fruits by using spray method

Effect of temperature on diameter growth rate (mm)

The results of the current study showed a direct effect of temperature on the growth of Alternaria mushrooms, as the growth rate of *Alternaria alternate* reached 7.83 at 28 ° C. While the lowest growth rate of *Alternaria destruens* was 2.33 at 4 ° C, as shown in figure (8).

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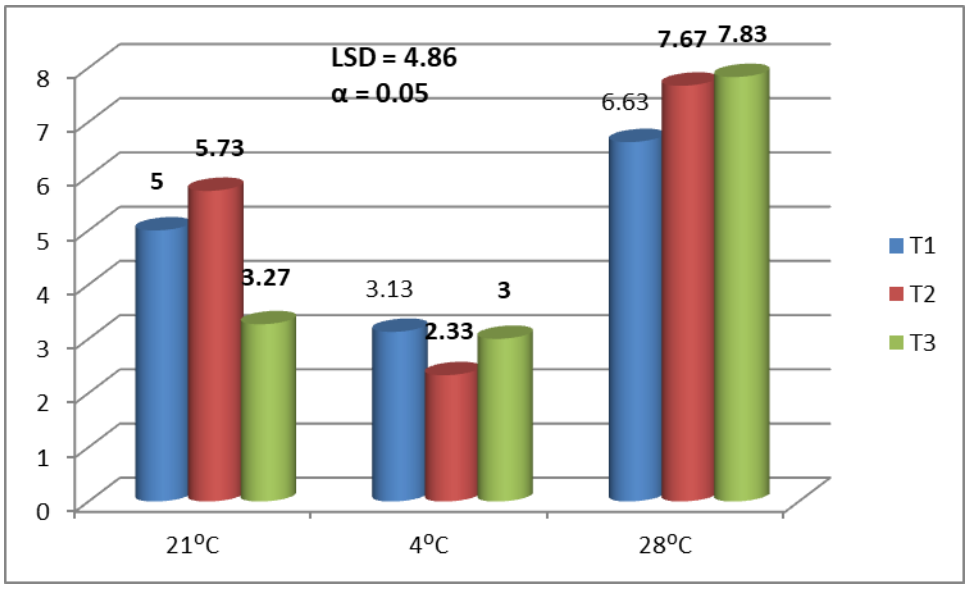

Figure (8): Effect of temperature on diameter growth rate (mm).

Molecular study

Within this locus, three samples were included, which had shown about 574 bp amplicons length. Before sending these ribosomal amplicons to sequencing, it was made sure that all the amplified amplicons had shown sharp, specific, and clean bands.

The sequencing reactions had indicated the confirmed identity of the amplified products by performing NCBI blastn. Concerning the 574 bp PCR amplicons of the ribosomal gene, the NCBI BLASTn engine showed entire sequences of similarities between the sequenced samples and *Alternaria alternata* sequences. NCBI BLASTn engine has indicated the presence of 100% of homology with the expected target that covered a portion of the 2 small subunit ribosomal RNA gene, entire internal transcribed spacer 1, 5.8S ribosomal RNA gene, and entire internal transcribed spacer 2, and partial large subunit ribosomal RNA gene. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. MT573464.1), the exact positions and other details of the retrieved PCR fragments were identified (Fig. 8).

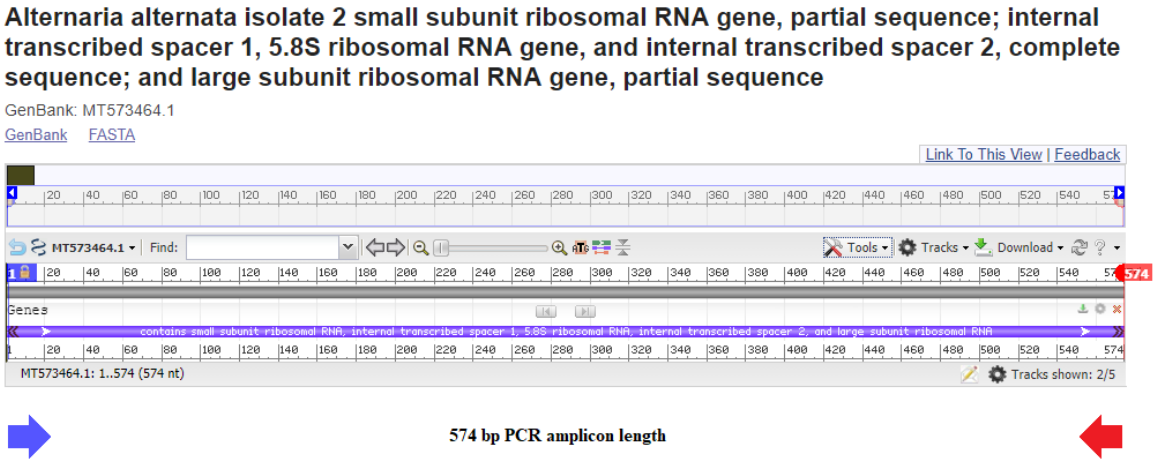

Figure (9): The exact position of the retrieved 574 bp amplicon that partially covered the ribosomal region within *Alternaria alternata* genomic sequences (GenBank acc. no.

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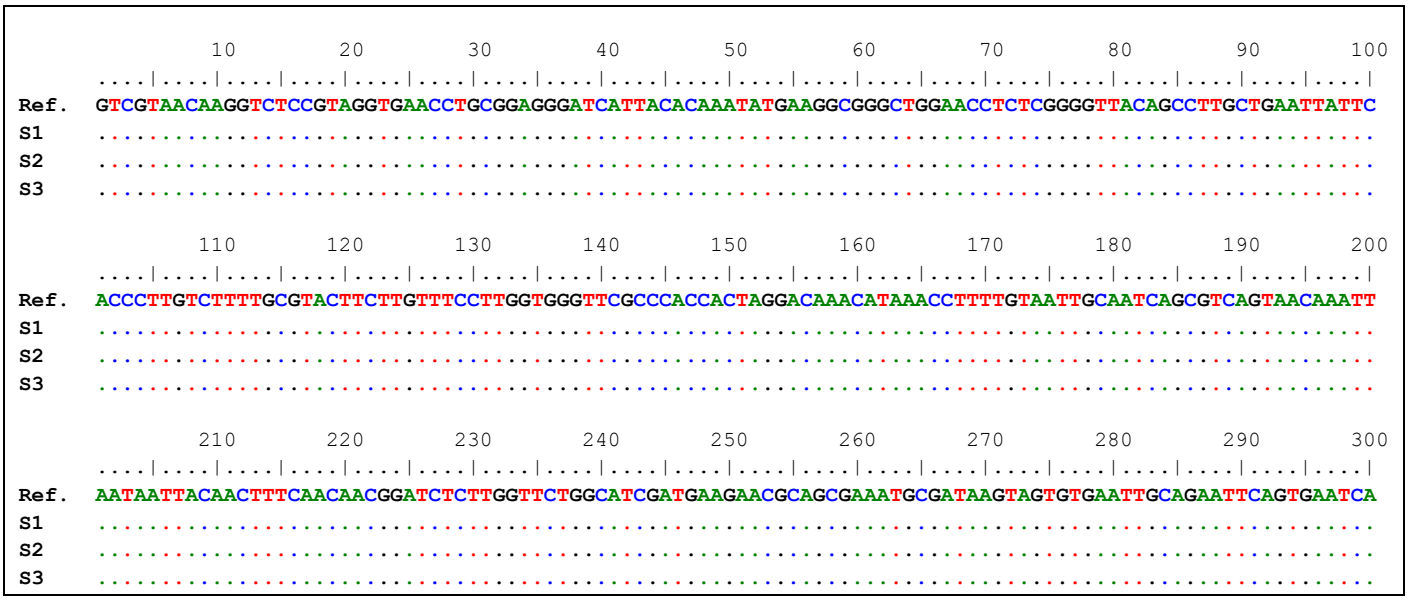
MT573464.1). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

After positioning the 574 bp amplicons' sequences within the ribosomal sequences, the details of these sequences were highlighted within the amplified ribosomal small subunit sequences (Table 1).

Table (2): The position and length of the 574 bp PCR amplicons that used to amplify a portion of the small ribosomal subunit within *Alternaria alternata* genomic sequences. The amplified sequences were extended from 1 to 574 of the NCBI reference sequence (GenBank acc. no. MT573464.1).

Amplicon	Reference locus sequences (5′ - 3′)	length
Small ribosomal sequences	GTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGA TCATTACACAAATATGAAGGCGGGCTGGAACCTCTCGG GGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCG TACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGAC AAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAAC AAATTAATAATTACAACTTTCAACAACGGATCTCTTGGT TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT AGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG AACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCC TGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGT TGGGCGTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTA AAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAG CACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCA TTAAGCCTTTTTTTCAACTTTTGACCTCGGATCAGGTAGG GATACCCGCTGAACTTAAGCATATCATA	574 bp

The alignment results of the 574 bp samples revealed the detection of no nucleic acid variations with the corresponding referring sequences of GenBank sequences (MT573464.1) (Fig. 9). These sequences were prepared by aligning with the GenBank acc. MT573464.1.



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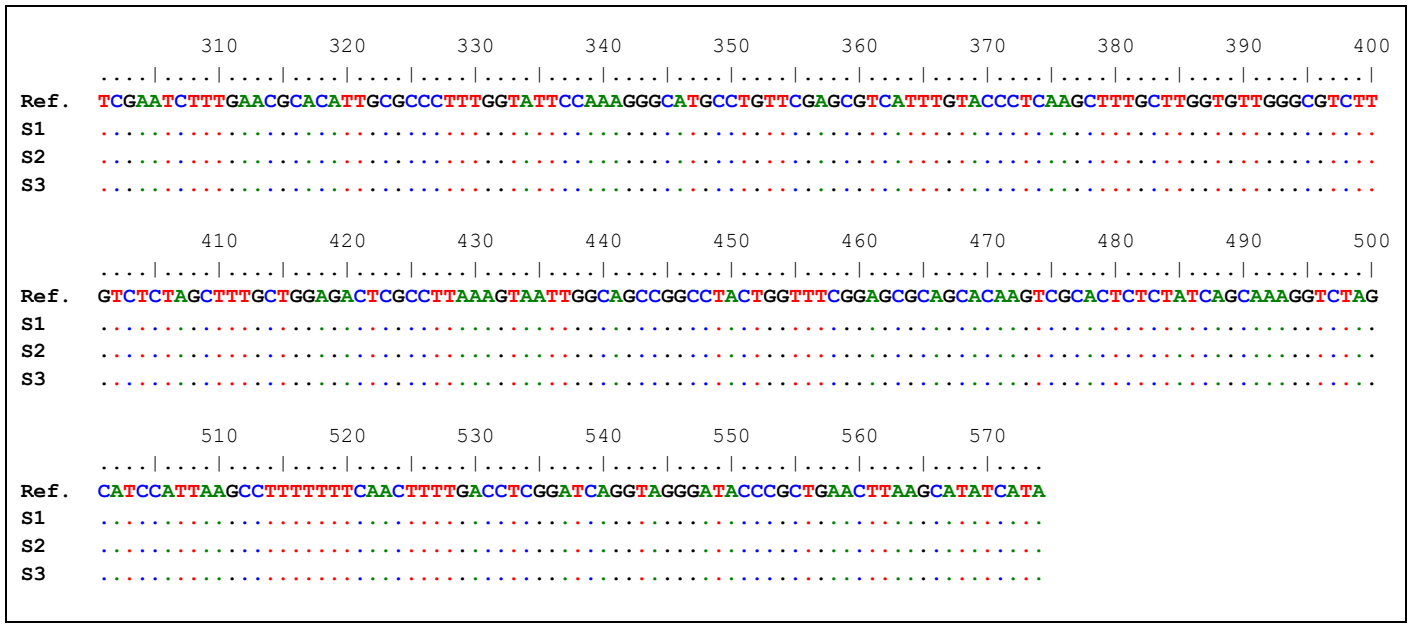

Figure (10): Nucleic acid sequences alignment of three local samples with their corresponding reference sequences of the small ribosomal subunit within the *Alternaria alternata* genomic sequences. The symbol "ref" refers to the NCBI reference sequences, while "S No.#" refers to sample numbers.

A comprehensive phylogenetic tree was generated, which was based on the observed nucleic acid sequences detected in the investigated fungal samples. Along with the other deposited DNA sequences, this phylogenetic tree contained all our currently investigated samples (S1 to S3 samples). A total number of the aligned nucleic acid sequences in this comprehensive tree was 90 sequences. This comprehensive tree indicated the presence of only one genus, *Alternaria*, which represents the only incorporated nucleic acid sequences within the tree. Based on the currently analyzed ribosomal sequences, *Alternaria* sequences were clustered into two main clades. The first one was represented by the incorporation of three different species of Alternaria, namely *A. alternata, A. destruens,* and *A. tenuissima*. This three species clade was the main observed groups within the generated comprehensive tree. Within this clade, our investigated samples were incorporated. Noteworthy, it was found that *A. alternata* had taken an immediate vicinity to our investigated samples, which entails more proven data for the close homology between our samples and this species. However, the currently analyzed ribosomal sequences showed extreme similarity between *A. alternata, A. destruens,* and *A. tenuissima* that shared the same clade. These observations indicated that these three different species of *Alternaria* had not exhibited any obvious differences in their 2 small subunit ribosomal sequences. In contrast to clade one species, the present tree revealed another clade, clade-2. Within this clade, only one type of fungal sequence was incorporated, namely *A. brassicicola*. This species exhibited a clear differentiation from the clade-1 species in terms of phylogenetic differences. The reason for this relatively distant position was attributed to the ability of these ribosomal sequences to differentiate *A. brassicicola* from *A. alternata, A. destruens,* and *A. tenuissima* the nucleic acid sequences. However, there was no deviation from *Alternaria* as these two groups were only minor variations within the sequences of these fungal sequences. Though two distinct nucleic acid variations were observed among the investigated samples that

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subdivided these samples into two groups, all these fungal groups were found to exhibit close phylogenetic associations since small tree scale distances were observed among all incorporated sequences (Fig. 4). However, the distinct role of the generated phylogenetic tree in the accurate detection of the currently investigated samples could not be ignored. Accordingly, this notion provided a further indication of the fungal identity and accurate genotyping of these local studied samples. This ribosomal -based comprehensive tree has provided an extremely inclusive tool about the high ability of such sequences to efficiently identify *Alternaria* samples using these genetic fragments.

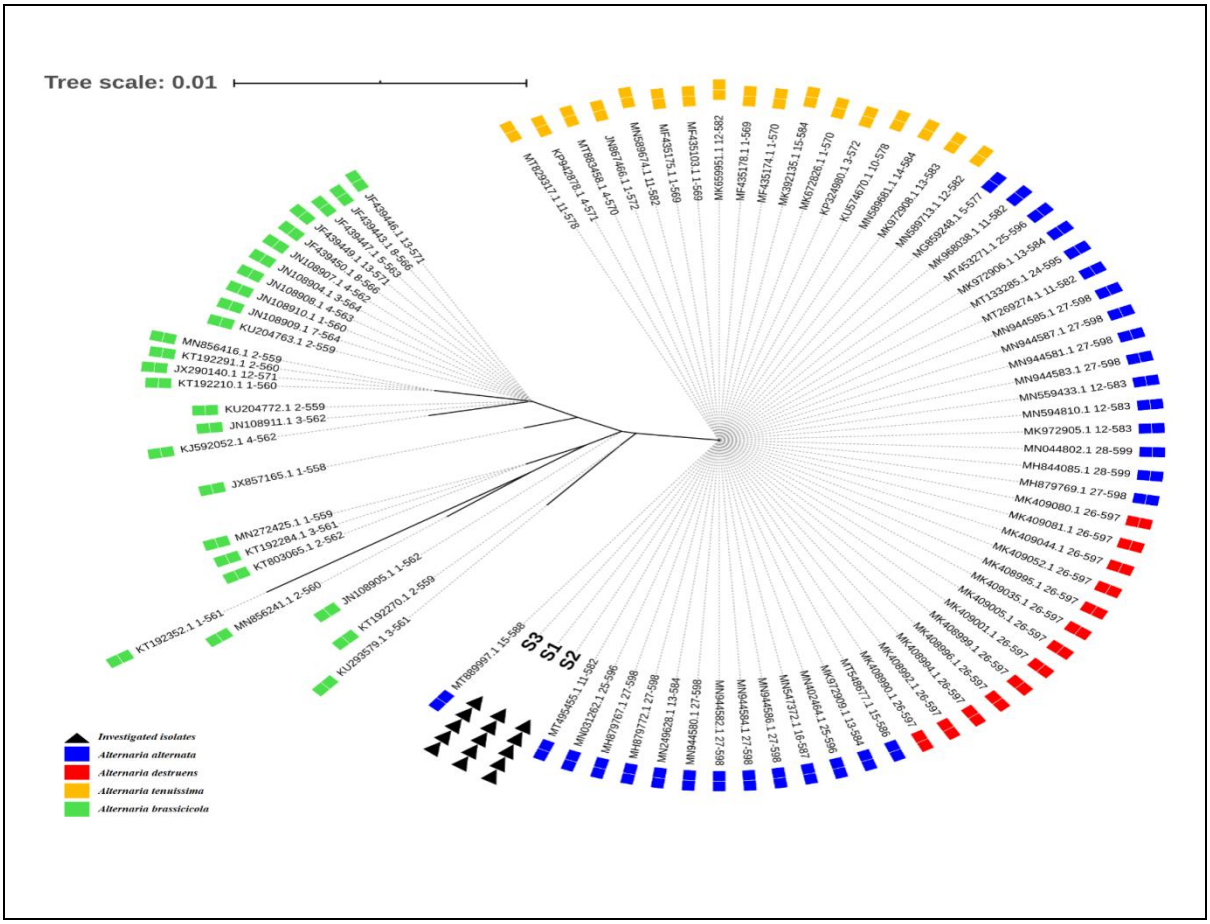

Figure (11): The comprehensive phylogenetic tree of the local isolates of the 574 bp amplicons that partially covered the ribosomal region within *Alternaria* genomic sequences. The variable colors refer to the variable grouping of the analyzed variants, within their Genbank deposited sequences. The number "0.01" at the top left portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The symbols S1 – S3 refer to the code of the investigated samples.

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