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Molecular Characterization of *Pythium* Spp. Isolated from Tomato Seedlings in the Syrian Coast

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Abstract: Tomato seedlings damping-off is a limiting factor in commercial greenhouse production. To determine the causal agents of disease, sampling and fungal isolation were performed during 2012. Samples were collected from infected seedlings growing in greenhouses in the Syrian coastal region. Isolation of fungi was done in the laboratories of the Agronomical Reaserch Center, in Lattakia and the molecular analyses were done in the Biotechnology Center at Tishreen University, Lattakia, Syria, during the years 2012, 2013. Eight isolates of *Pythium* sp. obtained were purified using hyphal tip method (named P1, P2, P3, P4, P5, P6, P7 and P8). Isolates were morphologically identified by optical microscope, then molecularly Characterized using genus specific ITS primers. The results of morphological characterization of pathogenic species suggested the detection of *Pythium aphanidermatum*, *P. ultimum*. The analysis of DNAs from the different isolates with ITS primers, recognizing the inter transcript spacer of nuclear ribosomal DNA proved that the eight, isolates were belonging to the species *P. ultimum*. The complete sequences of ribosomal DNA internal transcribed spacers regions of selected isolates were determined and submitted to GenBank. The GenBank-BLAST homology search revealed *P. ultimum* as the most similar sequence (> 96% identity) with GenBank entry AB355596.

Key words: Tomato, *Pythium* sp., Polymerase Chain Reaction (PCR), ITS.

1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) ranks as the leading fresh and processed vegetable crop in Syria and many other countries in the world. The number of greenhouses are approximately 67,977 (Statistical Abstract of Syrian Agriculture, 2012). It is grown practically all over the world in open fields, greenhouses and net-houses. However, tomato is a host for several pathogens including fungi, fungal like organisms, bacteria, viruses and nematodes. The environmental conditions that are favored to tomato (especially in greenhouse which always has high humidity and moderate to high temperature) enhance the conditions for the growth of specific soil-borne pathogens including the two fungal genus *Pythium* and *Phytophthora* (Kerkeni et al., 2007; Akaza *et al*., 2009). *Pythium* causes seed rot, damping-off and root rot which are the most destructive and economically important agricultural problems worldwide in nursery and greenhouse crops (Agrios, 2005). *Pythium* species are widely distributed in the world, and they are the major pathogens causing damping-off of seeds and seedlings (Kageyama and Nelson, 2003). The most important species of genus *pythium* sp. are prevailing in Syria and causing damping-off of tomato seedlings.

Pythium aphanidermatum (Edson) Fitzp, *pythium ultimum* Trow (Al-Shaabi *et al*., 2007). Seeds infected with this fungal pathogen typically won't germinate and will decompose. Young seedlings may topple over and their stems develop mushy lesions. Water-soaked lesions appear on fruits infected with *Pythium* spp. and infected tissues such as stems and roots will begin to darken and become soft before

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rotting. Another symptom of *Pythium* and root rot is a white mycelial growth with a cotton-like appearance forming on infected plant tissue. Some infected tomato plants may have stunted growth while others show few symptoms above ground (Agrios, 2005). The genus *Pythium* is belonging to the family of Pythiaceae, order Perenosporales, class Oomycetes, phylum Heterokonta (Dick, 2001). More than 200 species of this genus have been described (Paul *et al.,* 1999). Historically, species identification of this genus was mainly based on morphological characteristics including the size and shape of oogonia, antheridia and sporangia on Corn Meal Agar CMA (Shahzad *et al*., 1990). The traditional identification brought about obscure delimitation between some species because they had few variable morphological characteristics or lost their ability to produce sexual structures (Levesque and Cock, 2004). In recent years, the morphological characterization has been supplemented by molecular characteristics of given species (Paul, 2004). Morphology-based taxonomy is increasingly being supplemented by molecular characteristics of a given species (Paul, 2004). The recent advent of molecular biology has contributed to the diagnosis of plant pathogenic fungi by offering new revolutionary methods for quicker and more accurate detection, identification and quantification. Fungi can be identified at the species level by primers designed on selected conserved sequences like the rRNA gene cluster followed by further characterization of the amplified fragment. The rRNA gene cluster became very popular for a number of reasons; it has several hundred copies per genome and it carries highly conserved and variable regions. Sequences of the rRNA subunits have been used for taxonomic and genetic studies, while conserved regions of the internal transcribed spacers (ITS) and the intergenic spacers (IGS) have been targeted for fungal detection (Paplomatas, 2006). Traditionally, the most prevalent techniques used to identify plant pathogens have relied upon culture-based morphological approaches. These methods, however, are often time-consuming, laborious, and require extensive knowledge of classical taxonomy. Other limitations include the difficulty of some species to be cultured in vitro, and the inability to accurately quantify the pathogen (Goud and Termorshuizen, 2003). These limitations have led to the development of molecular methods with improved accuracy and reliability. A high variety of molecular methods have been used to detect, identify and quantify a long list of plant pathogenic fungi. Molecular methods have also been applied to study the genetic variability of pathogen populations, and even to describe new fungal species. In general, these methods are faster, more specific, more sensitive, and more accurate, and can be performed and interpreted by personnel with no specialized taxonomical expertise (Levesque and Cock, 2004). The PCR for the amplification of the ribosomal gene has been used for the genetic identification of many organisms because it comprises both highly conserved sequences during evolution and highly variable sequences among species and even within species. Plenty of studies on molecular phylogeny among *Pythium* species and even within *Pythium* species have been conducted (Schurko *et al*., 2003; Lévesque and de Cock 2004) Molecular phylogenies based on the ITS region have been recently produced for the genus *Pythium* (Matsumoto et al., 1999; Levesque and de Cock, 2004). The Internal Transcribed Spacer (ITS) region of the rRNA gene sequences has become a useful tool for *Pythium* taxonomy, and it can also be used for identifying or detecting different *Pythium* spp. (Paul, 2000, Levesque and de Cock, 2004).

2. Material and Methods

2.1 Morphological Identification

Infected seedlings were collected from many tomato greenhouses in the Syrian coastal regions. Preliminary identification of fungi species in the eight different cultures was done using morphological

Characters of spores. Morphological Characters showed that the eight isolates belong to the two species: *Pythium aphanidermatum* (Edson) Fitzp, *pythium ultimum* Trow (Khrieba *et al*., 2013).

2.2 Molecular Identification

2.2.1 DNA isolation

Eight isolates (P1, P2, P3, P4, P5, P6, P7 and P8) were sub-cultured on vegetable juice medium (V8). Each isolate was sampled and inoculated in 25 ml of V8 containing 2.5 g of $CaCO_3$, then incubated in a shaker incubator at 28 °C with speed of 120 rpm for ten days. After ten days of incubation under darkness at 28 °C, the fungal mycelium from the eight isolates were harvested and dried on absorbent paper. DNA was extracted from mycelium using CTAB protocol. (Benito *et al*. 1993).

2.2.2 DNA amplification by Polymerase chain reaction (PCR)

DNAs of different isolates were analyzed using Opmycete ITS primers. The primer pair ITS1/ITS4 [ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3')] and primer pair ITS6/ITS4 [ITS6 (5'-GAA GGT GAA GAA GTC GTA GTA ACA AGG-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3')] were used to distinguish the isolates of *Pythium* genus (White *et al*., 1990) where it amplifies a single band of 900 bp (Cooke *et al*., 2000). The specific primer pair puk1/puk2 [(Puk1 F: '5-ACGAAGGTTGGTCTGTTG-3') and (OOMS-lo 5.8 47 B R: '5- TCTTGTCTGATATCAGGTCg-3')] were used to differentiate isolate belonging to *P. ultimum*, while the specific primer pair P18GAA1-71/P18GAA1-71 amplify a specific product in *P. aphanidermatum* (Lee and Moorman, 2008). The specific primer pair P18GAA1-71/P18GAA1-71 [(P18GAA1-71 F: '5-CGCATTACGTATCGCAGTTCGCAG-3') and (P18GAA1-71 R: '5-ATTCAGTTTATCACAGCGACGA-3')]. The PCR reaction was performed in 20 µL containing 20 ng of DNA (4 µL), 2 µl of each primer (100 µM), 10 µL GoTaq Green Master Mix (Promega BioSciences, LLC, San Luis Obispo, CA, USA), 4µL of H2O. Amplification was performed in an eppendorph thermal cycler. The PCR program started by an Initial denaturation at 95 °C for 4 min, followed by 35 cycles, each of them is composed of a denaturation step at 95 ºC for 1 min, annealing step at 55 °C for 1 min, and an extension step at 72 °C for 70 sec. and one cycle for a final extension step at 72 °C for 5 min. The amplified products were separated on 1.2% agarose gels in a 1X TBE buffer. Gels were stained ethidium bromide and were photographed under UV light.

2.2.3 Sequencing PCR products

Direct sequencing of the PCR products was done by the DNA sequencing laboratory of Biotechnology in National Center for Biotechnology (NCBT). The complete rDNA ITS regions were sequenced in both directions using the primers ITS1 and ITS4. DNA sequence data obtained in this study has been deposited in GenBank. DNA sequences were compared to homologous sequences registered in GenBank using the standard nucleotide-nucleotide BLAST protocol (http://www.ncbi.nlm.nih.gov/BLAST/).

3. Results and Discussion

3.1 Molecular Characterization

3.1.1 Amplification and characterization of the rDNA ITS sequences

PCR amplification of the rDNA ITS region of Pythium isolates using the ITS6-ITS4 primer pairs was successful and gave PCR products of 900 base pair (bp). Use of the primers ITS1 and ITS4: 910 bp showed that all isolates belong to the genus pythium sp. (Fig. 1). Amplification of the target P. ultimum DNA was done using the specific primers Puk1and puk2 band and gave approximately 670 bp. But, amplification of the P. aphanidermatum DNA was done using the specific primer pair P18GAA1-71F

and P18GAA1-71R, giving no bands (Fig. 2)

The rDNA ITS nucleotide sequences of those isolates showed high sequence homology (> 96% identity) (Fig. 3). The GenBank-BLAST homology search using these ITS sequences revealed *P. ultimum* as the most similar sequence (> 96% identity) with GenBank, and thus show that the eight isolates belong to *P. ultimum*.

4. Discussion

The identification of most *Pythium* species is difficult due to overlapping of morphological characteristics, as clearly illustrated in the Venn-diagram taxonomic key of Dick (1990). Moreover, cultural conditions can influence the variability of morphological characteristics and lead to misidentification of species (Kageyama *et al.,* 2005). The result of failure could be in identification of some characters of *Pythium*. Therefore, phylogenetic relationship based on molecular identification was used to determine the real name of the pathogenic fungi. Because of reliance of many recent phylogenetic analyses on sequences of the internal transcribed spacer ribosomal DNA (ITS rDNA) region, the DNA sequence database in GenBank is comprehensive, and sequencing has become a powerful tool for identification (Lévesque and de Cock 2004, Matsumoto et al 1999, Villa et al 2006). At present, amplification of DNA sequence by primers ITS5 and ITS4 is similar to the results that always produce a single band approximately 900 bp as obtained Cooke et al. (Cooke *et al.* 2000). Target DNA consisted of small subunit (SSU) 18S partial sequence, the ITS1, ITS2, 5.8S rDNA complete sequence, and large subunit (LSU) 28S rDNA partial sequence (Matsuda et al, 2005). Molecular biology has impacted more and more heavily on mycology (Borman *et al*, 2008). Species identification based on phenotypic identification is often time-consuming, requiring high attention by researchers, having high error probability (Siricord, 2005) and is readily influenced by cultural conditions (Borman *et al*, 2008). Conversely, molecular phylogenetic identification involving

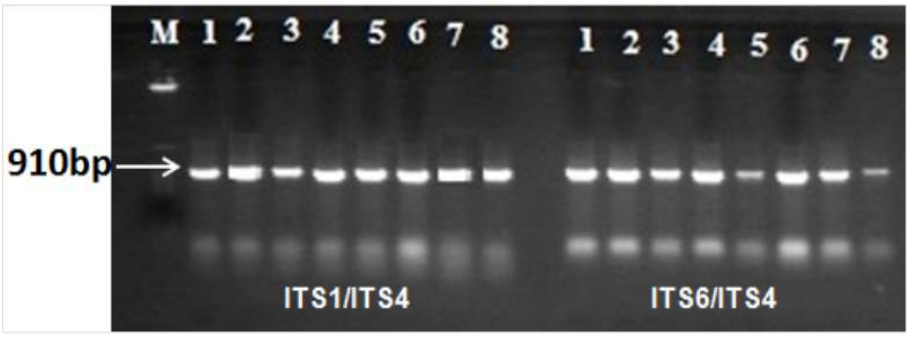

Fig. 1 Extracted DNA of *Pythium* sp was amplified with primer pairs ITS 1 and 4, ITS 4 and 6, and M is a marker (GeneRuler™ DNA Ladder Mix 100).

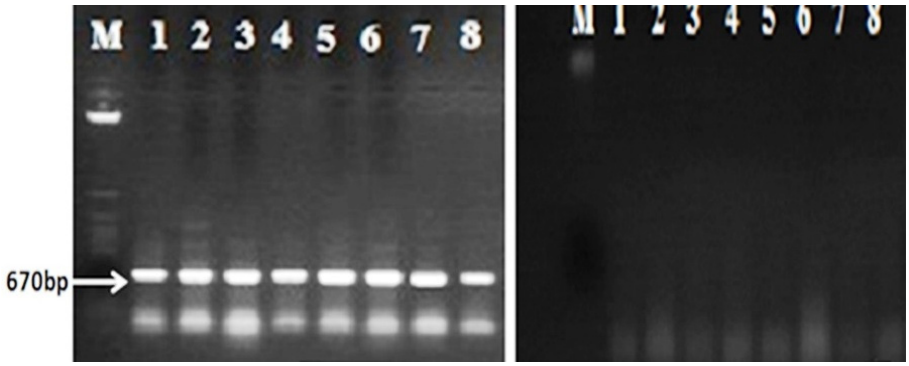

Fig. 2 Amplification of the target *P. aphanidermatum* DNA was done using the specific primers P18GAA1-71F, P18GAA1-71R (1). (2) Amplification of the target *P. ultimum* DNA was done using the specific primers Puk1and puk2.

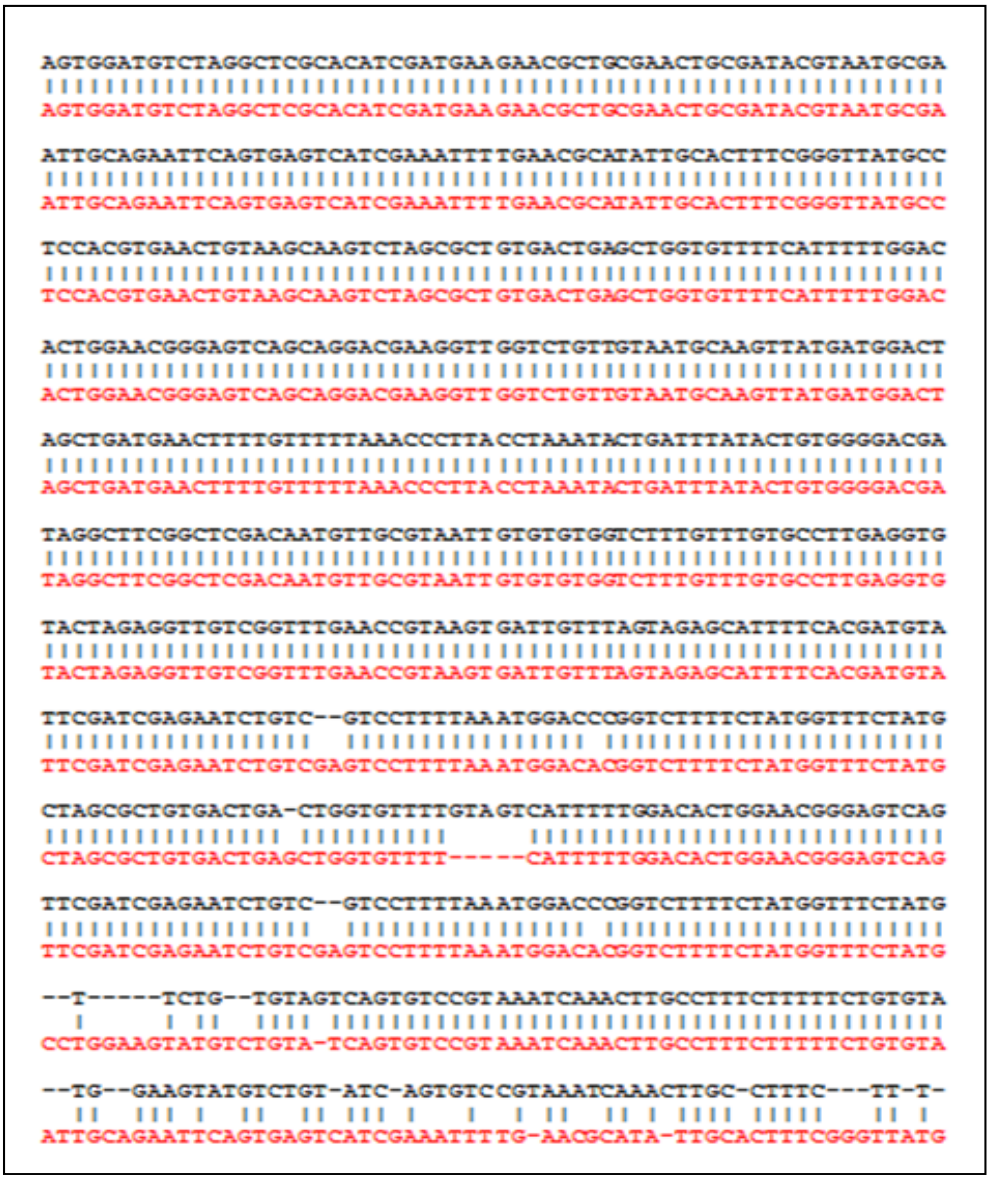

Fig. 3 Nucleotide sequences to one of the isolates fungus Pythium ultimum (top) with isolation AB355596 (bottom) recorded in the gene bank.

gene sequencing is more objective, uninfluenced by growth conditions, and capable of discriminating between fungi that fail to produce distinctive morphological features (Borman *et al*, 2008).

5. Conclusions

The pathogenic species identified based on morphological characterization were close to *Pythium aphanidermatum, P. ultimum var. ultimum, and P. paroecandrum.* Their pathogenicity was evaluated on tomato seedlings under greenhouse conditions. All isolates were pathogenic. This is the first report on occurrence of Pythium species causing seedlings damping off in greenhouses in the coastal region. Molecular methods have been used to analyze ITS region of the nuclear ribosomal DNA. Moreover, molecular characterization using Polymerase Chain Reaction (PCR) showed that the eight isolates belong to the species *P. ultimum*.

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