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MOLECULAR IDENTIFICATION OF NOVEL ISOLATES OF *RHIZOCTONIA SOLANI KÜHN* AND *FUSARIUM* SPP. (MATSUSHIMA) ISOLATED FROM PETUNIA PLANTS (*PETUNIA HYBRIDA* L.)

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Abstract

This study was conducted in the Plant Virology Laboratory, College of Agriculture, Karbala University with the aim of isolating and identifying some fungi, isolated from petunia plant (*Petunia hybrida* L.) roots collected from some private nurseries in Najaf province. The polymerase chain reaction (PCR) technique was used to determine the nucleotide sequence of PCR-amplified products. The results from the PCR amplification and analysis of the nucleotide sequences of PCR-amplified products by using BLAST program (Basic Local Alignment Search Tool) showed that the isolated fungi are belonged to *Rhizoctonia solani*, *Fusarium verticilliodes*, *Fusarium proliferatum*, *Fusarium solani* and *Trichoderma harzianum*. By comparing the nucleotide sequences generated from PCR products amplified from the ITS1-ITS4 region of the above-mentioned fungal isolates with the nucleotide sequences, belonged to the same fungi and available in the National Center for Biotechnology Information (NCBI), it was found that the identified fungal isolates of *R. solani*, *F. verticilliodes, F. proliferatum* were not previously recorded in NCBl, therefore; the identified sequences of *R. solani*, *F. verticilliodes, F. proliferatum* have been deposited and recorded in GenBank database (NCBI) under the accession numbers : KX828175, KX828174 and KX828173.

Key words : PCR technique, *R. solan, F. verticilliodes*, *F. proliferatum, Petunia hybrida*.

Introduction

Fungi is the second largest group of eukaryotic organisms on earth, with an estimated number ranging from 1.5 to 5.1 million species identified up-to-date (Hawksworth, 1991; O'Brien *et al*., 2005). A large number of fungal species are pathogenic to plants and a smaller group pathogenic to animals and on human (Hawksworth *et al*., 1995; Magg *et al*., 2013). Many soil-borne plant pathogens, for instant *Rhizoctonia solani*, *Pythium* spp. and *Fusarium* spp., can cause a negative impact on the quality and productivity of the crops in the world (Amatulli *et al*., 2010; Shin *et al*., 2014).

Identification of fungi for species level is paramount in both applied (genomics, bioprospecting) and basic (ecology, taxonomy) applications in scientific research, especially for natural products researchers working with fungi as a source of bioactive secondary metabolites (Raja *et al*., 2017). Scientific names of fungi enable researchers to identify other closely related species to a better predictionof the evolution of chemical gene clusters (Schmitt and Barker, 2009) or to prioritize taxonomically associated strains, when a productive strain may attenuate production of key bioactive compounds (Sudhakar *et al*., 2013). More importantly, taxonomic identification of fungi is extremely essential when industrial as well as agrochemical, or pharmaceutical products are to be derived from a fungal isolate.

Fungal identification based on morphological characteristicsis time consuming and requiresan excellent experience in identification of fungi, especially when dealing with closely related species, for example *Fusarium proliferatum*, *Fusarium fujikuroi*, *Fusarium saccharin*, *Fusarium subglutinansas*, *Fusarium verticillioides* and *Fusarium andiyazi*, in addition to its need for time and effort (Yang *et al*., 2007; Wang *et al*.,

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2008; Zhang *et al*., 2012 and Huang *et al*., 2016). Identification of fungi using morphological characteristics could lead to incorrect species designation; therefore, there is an urgent need to develop and use methods for rapid, sensitive and specific diagnosis. Nucleic acid (NA) based techniques such as polymerase chain reaction (PCR)are powerful for plant pathogen detection and gives a reliable and sensitive microbial identification when applied during surveillance programs (Kim *et al*., 2008). Molecular approaches based on DNA analysis can be used to detect genetic diversity of fungi and display the potential benefits of highly sensitive and quick detection (Saad *et al*., 2004). Molecular identification of fungifor species level has been based mainly on the use of variable rDNA (ribosomal-DNA) internally transcribed spacer (ITS) regions that has been foundwith highly variable sequences and serve as reliable markers for taxonomically more distant groups of fungi (Anderson *et al.,* 2003; Lord *et al.,* 2002).

The rapid and accurate identification of a pathogenic fungal species is important to achieve protection of crops and other natural resources by developing an efficient disease control management, useful quarantine purposes and making correct decisions. In the present study, the PCR techniquewas used to characterize the fungi *R. solani, F. verticilliodes, F. proliferatum*, *Fusarium solani* and *Trichoderma harzianum* and the nucleotide sequences of the PCR-amplified ITS1 and ITS4 regionswere determinedto identify the genetic variations between these fungal isolates and the otherabove mentioned isolates previously published in the National Centre for Biotechnology Information (NCBI).

Materials and Methods

Fungal isolation and culture conditions

Fungal species in this study were isolated from roots of petunia plants sampled from some private nurseries in Najaf provincem, Iraq. Each sample was washed with running tap water for about one hour to remove the suspended dust. Then, it was cut into small pieces and surface sterilized for 5 min with 1% sodium hypochlorite solution (Clorox) for two minutes. After that, the samples were rinsed with sterile distilled water for three times and dried on sterilized filter papers to remove excess water before placing on Petri-dishes containing Potato Dextrose Agar (PDA) supplemented with chloramphenicol antibiotic (200 mg/L).

After 48-72 h incubation at 25±2°C, hyphae from the margin of each appeared colony were repeatedly transferred to other PDA medium plates until getting pure fungal growth that can be used for DNA extraction and identification. The identity of each isolated fungus was determined using the RCR technique and the DNA sequencing as described below.

DNA extraction and rDNA-ITS region amplification

Total genomic DNA was extracted from 5-7 days old colony of each fungal isolate using the plant genomic DNA extraction kit (Favorgen, Cat. No: FAPGK100) following the manufacturer's instructions. About 100 mg of fresh mycelia of each isolate already grown on PDA were scraped with a sterile scalpel and transferred into Eppendorf tubes for DNA extraction. The quality and quantity of extracted DNA was measured by a UV spectrophotometer (Thermo Scientific, Germany) and DNA was then stored at -20 until use.

PCR amplification was performed using *Taq* DNA polymerase (Roche, Cat. No. 11 146 173 001) in a 20 µl PCR reaction mixture containing 2 µ1 10X PCR buffer, 1 µl each primer (10 pmol), 3 µl template DNA (30 ng/µ1), 2 µl dNTPs (2 mM) and 1 unit *Taq* polymerase. Sample volume was adjusted to 20 µl with nuclease-free water. The ITS region of DNAs extracted from fungal isolates were amplified with the primer set of ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) (White *et al*., 2000). The DNA thermal cycler (Techne TC-5000, UK) was programmed for one cycle of 5 min at 94^0C, followed by 35 cycles of 1 min at 94^0C, 40 min at 55^0C and 40 min at 72^0C and one cycle of 5 min at 72^0C. PCR-amplified products were separated on 1.5% agarose gel stained with ethidium bromide, visualized under UV transillumination and pictures were then captured.

Before DNA sequencing, remaining dNTPs and primers were removed from the PCR product using the PCR Clean-Up Kit (Cat. No.: FAPCK 001, Favorgen, Taiwan). Purified-PCR products were sent along with the forward and reverse primers (ITS1 and ITS4) to Macrogen company (http://www.bionity.com/en/) to pinpoint the nucleotide sequence in both directions of each PCR product. All obtained nucleotide sequences were checked and analyzed using BLAST software (Basic Local Alignment Search Tool) and compared with the sequences previously published in NCBI database (Zhang *et al*., 2012).

Results and Discussion

Results showed the possibility of amplifying an approximately 500 bpPCR productfromDNA extracted from each isolated fungus and amplified using the primer pair ITS1 and ITS4 (fig. 1). A search BLAST was done

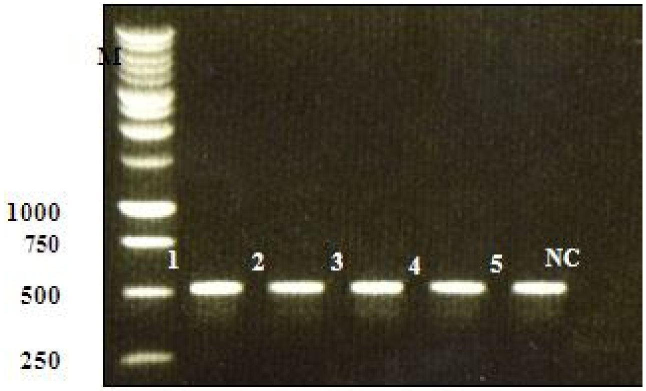

Fig. 1 : Ethidium bromide-stained agarose gel shows approximately 500bp PCR productsof internal transcribed spacer (ITS) region of *R. solani* (1); *F. verticilliodes* (2); *F. proliferatum* (3); *T. harzianum* (4); *F. solani* (5). M= 1Kbp DNA ladder marker. NC: Negative control (no template DNA added).

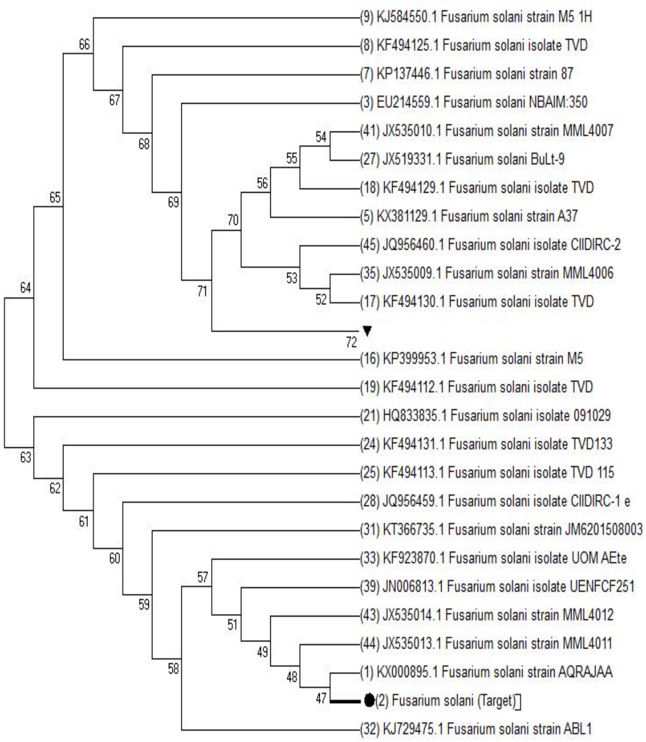

Fig. 2 : A phylogenetic tree generated using the neighbor-joining method based on a comparison of the whole ITS (ITS1, 5.8S rDNA, and ITS4) region sequence from the *F. solani* isolate used in this study, indicated by black dote (●), with those of other *F. solani* isolates available in GenBank (NCBI).

to nucleotide sequencesgenerated from the PCR-amplified products indicating that the identified fungi are belonged to the fungal species *R. solani*, *F. solani*, *F. verticilliodes*, *F. proliferatum* and *T. harzianum*.

A BLAST search on the nucleotide sequence generated from the PCR product amplified from *F. solani* isolated in this study indicated to an entirely identical (100%) to the Iraqi *F. solani* isolate (Accession No.: KX000895.1) previously isolated from corn plants. It was also noticed that this isolate gave a similar percentage which reached 99% with the other *F. solani* isolates identified in different geographic regions of the world and registered in NCBI (table 1, fig. 2).

It was also observed that the genetic similarity for ITS-amplified region of *T. harzianum* isolated in the current study was 100% to some of *T. harzianum* isolates ((Accession No.: KU317848.1, KU317846.1, KF986661.1 and KF986660.1) available in NCBI (table 2, fig. 3).

From comparison, results also indicated that the highest genetic similarity for ITS-generated sequence of *R. solani* isolated in the study was observed with the Iraqi *R. solani* isolate (99%, Accession No.: KF372660.1), followed by other *R. solani* isolates previously isolated from Iraq (Accession No.: KF372662.1, KF372646.1, KF372653.1, KF372645.1 and KF372657.1). The lowest genetic similarity was found the *R. solani* isolate identified in USA (Accession No.: FJ746906.1) that had a genetic similarity of 88%. It also noticed that that the genetic differences based on the ITS-sequenced region the *R. solani* used in this study ranged between 90-95% with those already identified *R. solani* isolates and published in NCBI (table 3, fig. 4).

As shown in table 4, comparison of the ITS-nucleotide sequence obtained from *F. verticilliodes* with the other *F. verticilliodes* isolates deposited in GenBank showed that the highest genetic similarity was 99% with the *F. verticilliodes* isolates obtained from China (Accession No. KT224787.1), India (Accession No. KM434131.1) and Malaysia (Accession No. KM396284.1). It also gave a 98% similarity with *F. verticilliodes* isolated from Kenya (Accession No. KM434131.1) (fig. 5).

The nucleotide sequence of PCR product amplified from *F. proliferatum* isolated in the current study showed an ITS-nucleotide sequence similarity of 97% with the other isolates belonged to the same fungal species and recorded in GenBank (table 5, fig. 6).

In conclusion, PCR technique was used in this study to identify the isolated fungi *R. solani*, *F. solani*, *F. proliferatum*, *F. verticilliodes* and *T. harzianum*. Among these isolates, it was proven, by determining and comparing the nucleotide sequence of PCR-amplified products with the ITS1 and ITS4 region already sequenced and registered in GenBank, that the fungal isolates *R. solani*, *F. proliferatum* and *F. verticilliodes* are novel isolates and not previously known; therefore,

Table 1 : Comparison between the nucleotide sequence similarity percentages of *F.solani* isolated in this study from petunia plants with the other isolates belonging to the same fungus and registered in NCBI.

Fungus	Isolate or strain name	Origin	The most similar sequences in GenBank database	
			GenBank Accession Number	Sequence similarity (%)
*F. solani**	-	Iraq	-	100
F. solani	AQRAJAA	Iraq	KX000895.1	100
F. solani	A11bs 10290 F7 18S	India	KP264956.1	99
F. solani	NBAIM:350	India	EU214559.1	99
F. solani	TVD_Fungal-Culture127	Canada	KF494125.1	99
F. solani	M5_1H	Hungary	KJ584550.1	99
F. solani	1A44 18S	China	KF572456.1	99
F. solani	Fs1 18S	India	KC156593.1	99
F. solani	CS11723 18S	China	JX406551.1	99
F. solani	bxq33104	China	EF534185.1	99
F. solani	AL1	China	KX650831.1	99
F. solani	Fso8	Tunisia	KU528858.1	99
F. solani	M5	China	KP399953.1	99
F. solani	TVD_Fungal-Culture132	Canada	KF494130.1	99
F. solani	TVD_Fungal-Culture131	Canada	KF494129.1	99
F. solani	TVD_Fungal-Culture114	Canada	KF494112.1	99
F. solani	CEF-325	China	KF999012.1	99
F. solani	091029 18S	China	HQ833835.1	99
F. solani	FUS ITS 11 18S	India	HQ384397.1	99
F. solani	Fso3	Tunisia	KU528851.1	99
F. solani	TVD_Fungal-Culture133	Canada	KF494131.1	99
F. solani	TVD_Fungal-Culture115	Canada	KF494113.1	99
F. solani	CrP21	India	KC920847.1	99
F. solani	CIIDIRC-1	Mexico	JQ956459.1	99
F. solani	bxq33102	China	EF534183.1	99
F. solani	Fso6	Tunisia	KU528855.1	99
F. solani	JM6201508003	China	KT366735.1	99
F. solani	ABL1	India	KJ729475.1	99
F. solani	UOM AE	India	KF923870.1	99
F. solani	TUFs8	Saudi Arabia	HG798753.1	99
F. solani	MML4006	India	JX535009.1	99
F. solani	JAI-MB22 18S	India	JQ954891.1	99
F. solani	BK-CB20 18S	India	JQ954888.1	99
F. solani	LCPANCF01	India	JN786598.1	99
F. solani	UENFCF251 18S	Brazil	JN006813.1	99
F. solani	GIFUUHFA10	India	GQ121291.1	99
F. solani	MML4007	India	JX535010.1	99
F. solani	P1 18S	India	GQ451337.1	99
F. solani	MML4012	India	JX535014.1	99
F. solani	MML4011	India	JX535013.1	99
F. solani	CIIDIRC-2	Mexico	JQ956460.1	99
F. solani	FS5 18S	Ireland	HQ265423.1	99
F. solani	FWC30	India	KU097265.1	99

**F. solani* isolated in this study.

Table 2 : Comparison between the nucleotide sequence similarity percentages of *T. harzianum* isolated in this study from petunia plants with the other isolates belonging to the same fungus and registered in NCBI.

Fungus	Isolate or strain name	Origin	The most similar sequences in GenBank database	
			GenBank Accession Number	Sequence similarity (%)
*T. harzianum**	-	Iraq	-	100
T. harzianum	Thar16	India	KU317848.1	100
T. harzianum	Thar14 18S	India	KU317846.1	100
T. harzianum	BHR2P1F3M	India	KF986661.1	100
T. harzianum	BHR1P1F2M	India	KF986660.1	100
T. harzianum	ZG-2-2-1 18S	China	KT192387.1	99
T. harzianum	ZG-2-4-1 18S	India	KT192396.1	99
T. harzianum	00003-1-1 18S	China	KT192339.1	99
T. harzianum	HNC21-106	China	KT959334.1	99
T. harzianum	A1 18S	China	KR708630.1	99
T. harzianum	BHR3P2F4M 18S	India	KF986662.1	99
T. harzianum	JSB301 18S	(Japan and China)	KC569359.1	99
T. harzianum	JSB22 18S	(Japan and China)	KC569353.1	99
T. harzianum	60503 18S	(Japan and China)	KC569348.1	99
T. harzianum	60574 18S	(Japan and China)	KC569346.1	99
T. harzianum	IIIC2b 18S	India	JX473720.1	99
T. harzianum	IVC3b 18S	India	JX473719.1	99
T. harzianum	VA1a 18S	India	JX473718.1	99
T. harzianum	IIA2a 18S	India	JX473716.1	99
T. harzianum	IIIA3b 18S	India	JX473715.1	99
T. harzianum	00111-1 18S	China	KT192196.1	99
T. harzianum	A2	China	KR708631.1	99
T. harzianum	CEN440	Brazil	KC576704.1	99
T. harzianum	61035	Japan	KC569350.1	99

* *T. harzianum* isolated in this study.

the identified fungal sequences have registered in Genbank under the accession numbers: KX828173, KX828174, KX828175, respectively.

In previous studies, PCR has widely used as a rapid and accurate technique in the diagnosis of many microorganisms, including plant fungi such as *Fusarium* spp., *R. solani* and *R. solani* to eliminate the restrictions of identification based on different morphological characters (Henry *et al*., 2000; Zakiah *et al*., 2016). Despite the usefulness of morphological charactersin sorting fungal isolates under identification into smaller groups before other methods of identification are applied, there are many limitations that accompany the identification of fungi such as the need for time and efforts, as well as the need for the sufficient experience in fungal identification, especially in relatively close fungal species (Leslie and Summerell, 2006; Yang *et al*., 2007; Wang *et al*., 2008; Zhang *et al*., 2012 and Huang *et al*., 2016). Other factors may also affect these morphological characters, such as moisture, light, type and composition of the growth medium, which can change the color, shapes and sizes of spores and fungal colonies developing (Zhang *et al*., 2012; Huang *et al*., 2016).

In Peninsular Malaysia, studies on *Fusarium* spp. is often based on morphological characters which could be led to incorrect species designation. In one of those studies, it was found that there are some limits on the use of morphological characters for the identification of some fungi such as species in the *G. fujikuroi* species complex as some species, for example, *F. proliferatum, F. fujikuroi*, *F. sacchari*, *F. subglutinans*, *F. verticillioides* and *F. andiyazi*, have very close morphological characters. It was observed through the re-diagnosis using the PCR technique that there is an error in the morphological identification of many fungi identified in previous studies such as species belonged to *Fusarium* spp., e.g., *F. verticillioides* and *Fusarium subglutinans* (Klittich *et al.,* 1997; Wulff *et al*., 2010

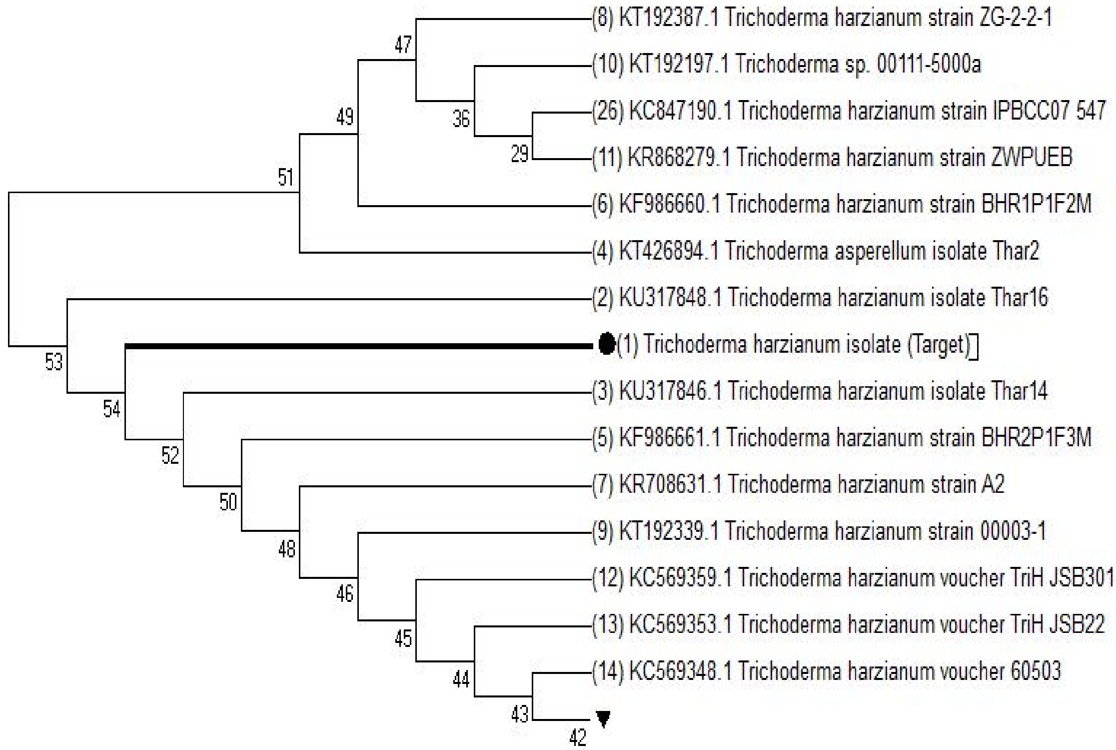

Fig. 3 : A phylogenetic tree generated using the neighbor-joining method based on a comparison of the whole ITS (ITS1, 5.8S rDNA and ITS4) region sequence from the *T. harzianum* isolate used in this study, indicated by black dote (•), with those of other *T. harzianum* isolates available in GenBank (NCBI).

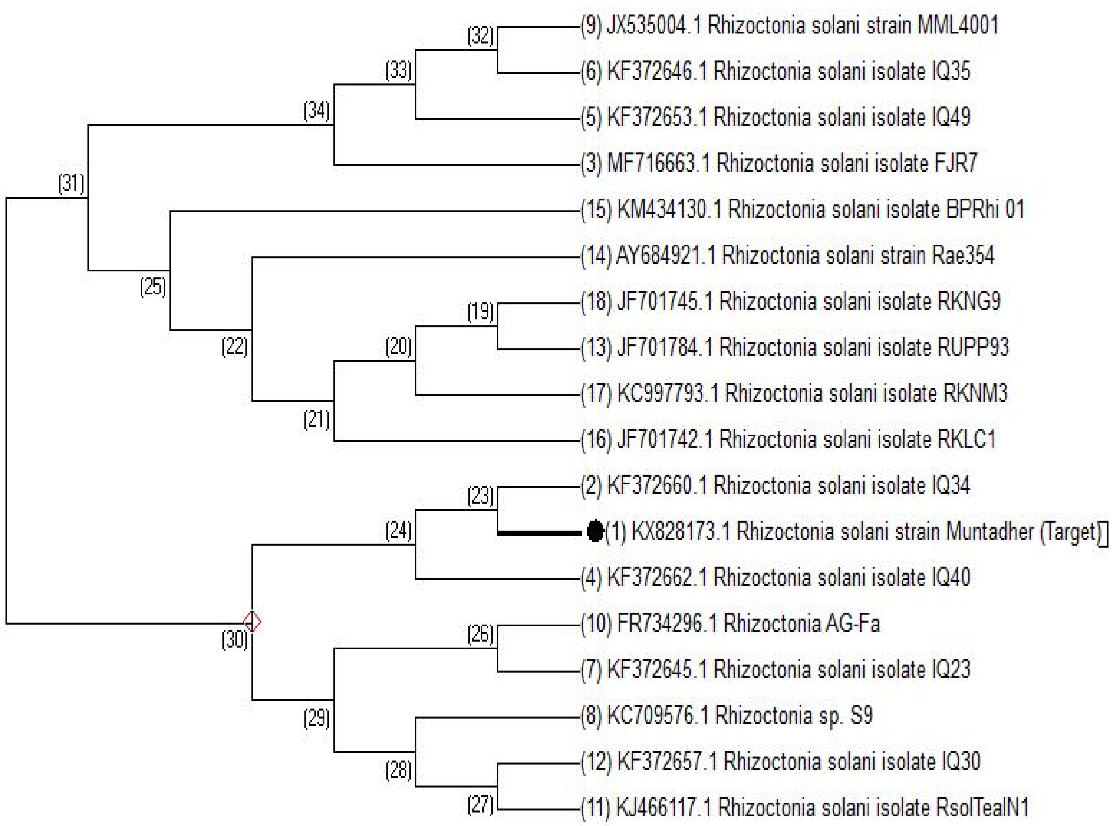

Fig. 4 : A phylogenetic tree generated using the neighbor-joining method based on a comparison of the whole ITS (ITS1, 5.8S rDNA and ITS4) region sequence from the *R. solani* isolate used in this study, indicated by black dote (•), with those of other *R. solani* isolates available in GenBank (NCBI).

Table 3 : Comparison between the nucleotide sequence similarity percentages of *R. solani* isolated in this study from petunia plants with the other isolates belonging to the same fungus and registered in NCBI.

Fungus	Isolate or strain name	Origin	The most similar sequences in GenBank database	
			GenBank Accession Number	Sequence similarity (%)
*R. solani**	Muntadher	Iraq	KX828173	100
R. solani	IQ34	Iraq	KF372660.1	99
R. solani	IQ40	Iraq	KF372662.1	96
R. solani	IQ35	Iraq	KF372646.1	95
R. solani	IQ49	Iraq	KF372653.1	95
R. solani	IQ23	Iraq	KF372645.1	95
R. solani	IQ30	Iraq	KF372657.1	95
R. solani	MML4001	India	JX535004.1	95
R. solani	RsolTeaIN1	India	KJ466117.1	95
R. solani	Rae354 18S	Taiwan	AY684921.1	93
R. solani	BPRhi 01	India	KM434130.1	93
R. solani	RUPP93 18S	India	JF701784.1	93
R. solani	RKNG9	India	JF701745.1	91
R. solani	RKLC1	India	JF701742.1	91
R. solani	RKNM3 18S	India	KC997793.1	91
R. solani	RDLM6	India	JF701717.1	90
R. solani	RT 5-1 18S	USA	FJ746906.1	88

**R. solani* isolated in this study.

Table 4 : Comparison between the nucleotide sequence similarity percentages of *R. solani* isolated in this study from petunia plants with the other isolates belonging to the same fungus and registered in NCBI.

Fungus	Isolate or strain	Origin	The most similar sequences in Gen Bank database	
			GenBank Accession Number	Sequence similarity (%)
*F. verticilliodes**	MUFAAQ1	Iraq	KX828175.1	100
F. verticilliodes	S9	China	KT224787.1	99
F. verticilliodes	BPFus 01 18S	India	KM434131.1	99
F. verticilliodes	UZ487 18S	Malaysia	KM396284.1	99
F. verticilliodes	CSB_F256	Kenya	KU680391.1	98

**F. verticilliode*s isolated in this study.

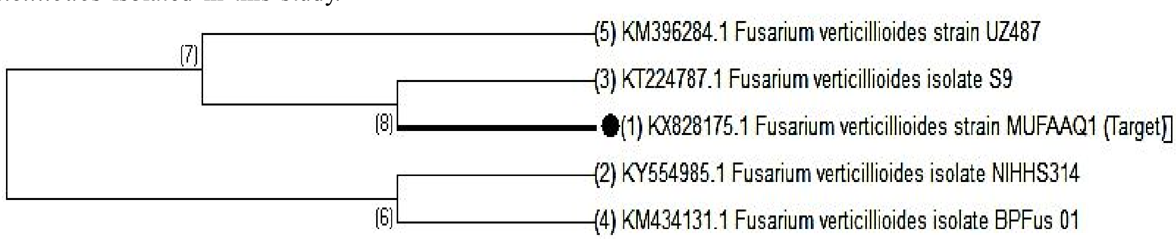

Fig. 5 : A phylogenetic tree generated using the neighbor-joining method based on a comparison of the whole ITS (ITS1, 5.8S rDNA and ITS4) region sequence from the *F. verticilliodes* isolate used in this study, indicated by black dote (Ï%), with those of other *F. verticilliodes* isolates available in GenBank (NCBI).

and Hsuan *et al*., 2011).

Differences in the Internal Transcribed Spacer (ITS) regions of the ribosomal DNA (rDNA), repeat units are well-investigated sequences that are existing in multiple copies and can be isolated and amplified by PCR (Alhussaini *et al*., 2016). PCR amplification of ITS region has been provided high efficiency in diagnosing many fungi such as *Fusarium* spp., *Cladosporium* spp. and *Fusarium verticillioides* (Chandra *et al*., 2008; Hsuan *et al*., 2011, Arif *et al*., 2012 and Alhussaini *et al*., 2016).

Table 5 : Comparison between the nucleotide sequence similarity percentages of *F. proliferatum* isolated in this study from petunia plants with the other isolates belonging to the same fungus and registered in NCBI.

Fungus	Isolate or strain name	Origin	The most similar sequences in GenBank database	
			GenBank Accession Number	Sequence similarity (%)
*F. proliferatum**	MUFAAQE2 5.8S	Iraq	KX828174.1	100
F. proliferatum	AF-04	China	MF426031.1	97
F. proliferatum	XJSFB 18S	China	KY968967.1	97
F. proliferatum	XJSF 18S	China	MF083155.1	97
F. proliferatum	1, 5.8S	China	MG562501.1	97
F. proliferatum	LrBF53	China	MG543771.1	97
F. proliferatum	LrBF52	China	MG543770.1	97
F. proliferatum	LrBF51	China	MG543769.1	97
F. proliferatum	LrBF49	China	MG543767.1	97
F. proliferatum	LrBF41	China	MG543759.1	97
F. proliferatum	LrBF36	China	MG543754.1	97
F. proliferatum	LrBF18	China	MG543736.1	97
F. proliferatum	LrBF10	China	MG543728.1	97
F. proliferatum	D73	China	KX878952.1	97
F. proliferatum	D66	China	KX878951.1	97
F. proliferatum	D39	China	KX878950.1	97
F. proliferatum	GA16	China	MF687307.1	97
F. proliferatum	M12	China	MF687294.1	97
F. proliferatum	El	China	MF686815.1	97
F. proliferatum	PR4	China	MF686808.1	97

* *F. proliferatum* isolated in this study.

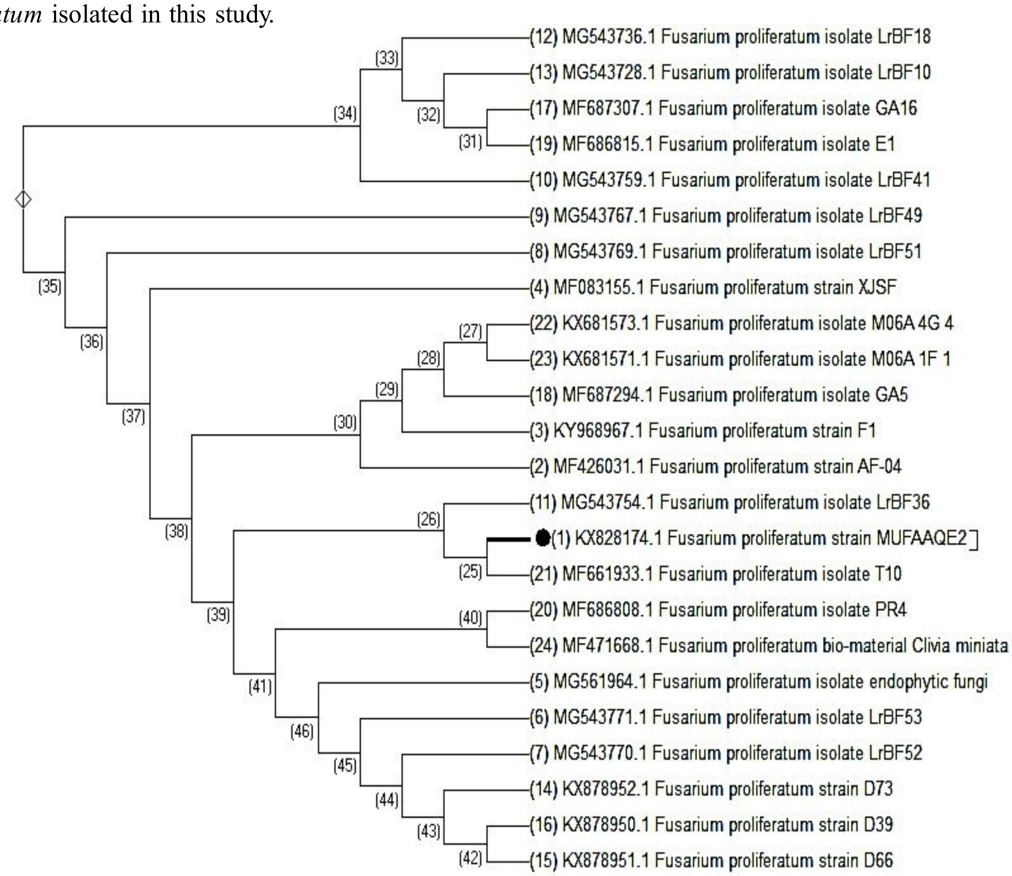

Fig. 6 : A phylogenetic tree generated using the neighbor-joining method based on a comparison of the whole ITS (ITS1, 5.8S rDNA and ITS4) region sequence from the *F. proliferatum* isolate used in this study, indicated by black dote (•), with those of other *F. proliferatum* isolates available in GenBank (NCBI).

The rapid and accurate identification of a plant pathogenic fungus is one of the urgent needs because of its importance for the development of effective disease control management, quarantine purposes and as a basis for makingcorrect decisions about cropsand other natural resources protection from fungal pathogens (Rossman *et al*., 2008).

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