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MORPHOLOGICAL, MOLECULAR AND PATHOGENICITY CHARACTERIZATION OF *PYTHIUM* SPP. ON ZUCCHINI ISOLATED FROM SOIL AND DISEASED PLANTS IN IRAQ

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Abstract

The study was conducted at Plant Protection Dept., College of Agricultural Engineering Sciences / University of Baghdad during 2018-2019. Twenty four isolates of *Pythium* spp. were isolate from different regions from diseased seedling and soil samples. Fourteen isolates recovered from seven regions in Baghdad and ten isolates from three regions in Al-Anbar. *Pythium* spp. were widespread in most of the cucurbits fields in the two provinces. Four media type (V8- A, PDA, CMA and CA) were capable of growing. The highest growth rate was on V8-A and the lowest growth rate was on CA media. All isolates were producing asexual and sexual structures. Sporangia were produce on CMA media within seven days at 25°C. Oospores were produce on V8-A and CA media after 14 day. The optimum temperature for all isolates growth was 30 and 25°C. The results of morphological characteristics show that S2 and S3 isolates were belong to *Pythium deliense* and this was the first report in Iraq. The other isolates were identified as *P. aphanidermatum*. Identification was confirmed by molecular characterization of isolates based on ITS region using ITS1&ITS4 primers. All 24 isolates sequences were deposited in the NCBI GeneBank database under the accession numbers (MN365079 - MN365100) and (MN365124 & MN365125). The phylogenetic tree of the 24 isolates in this study and other reference species from GeneBank were designed based on the distance method (neighbor-joining) using MEGAX program. Variations among the isolates were found. Also Iraqi isolates were differed from GeneBank isolates. Pathogenicity test for 24 isolate show that the two species were pathogenic on Zucchini and all the isolates were differed in pathogenicity causing pre and post emergence damping – off. Pre emergence was 10 – 52% and post emergence was 11 – 41%. The pathogenicity of *P. deliense* and *P. aphanidermatum* isolates were confirm for the first time on Zucchini.

Key words : Zucchini; *Pythium aphanidermatum; P. deliense*; First report; variation.

Introduction

The genus Pythium "fungal-like-organisms" or "pseudo fungi" was classified under kingdom Straminopila (Webster and Weber, 2007) or Chromista, phylum Oomycota, Class Oomycetse, Order Pythiales, Family Pythiaceae (Kirk *et al.,* 2008). It was first identified by Pringshim at 1858 including more than 230 species (Mathew *et al.,* 2003). Species of *Pythium* occur as saprophytes or parasites in soil, water, on plants, fungi, insects, fish, animal and human (Ho, *et al.,* 2012). The genus is one of the most important pathogen that attack plants and causing seed decay, pre and post damping off (Agrios, 2005). Also it hit the mature plants (fruit and vegetable) near harvest and post harvest, when they are on the markets.

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In Iraq *Pythium aphanidermatum* (Edson) Fitz is a very important pathogen causing economic losses (Al-Gaphagy, 1985). It was first repot by Al-Doory *et al.,* 1959 also it was isolated form sugar beet root (Dewan, 1977) and was reisolate from cucumber root on (1979) by El-Behadle & Al-Azawi. Later, this species has been widely studied in Iraq. In the other hand many studies were indicated that additional unidentified *Pythium* spp. are involved in the soil worldwide especially in the countries nearby Iraq. Four species, *P. aphanidermatum*, *P.spinosum P. splendens P. oligandrum* have been isolated from greenhouse that planted with cucumber in Oman (Al-Sa'di, *et al.,* 2007). *P. deliense* has been related to cause low production of Watermelon in Oman, the pathoginicity has been not published (Deadman *et al.,* 2007). In Egypt *P. aphanidermatum and P. diclinum*

were found on wheat root (Sheikh, 2010). Three species were identified P. aphanidermatum, P. ultimum P. deliense from cantaloupe plants that causing stem and root rot in Iran (Teymoori, *et al.,* 2012). Strong pathogenic *P. deliense & P. aphanidermatum* were isolate from soil that collected from car's tires which came from Egypt to Duba seaport in Saudi Arabia (Al-Sheikh, 2015). To date, as a result of a survey study three species were recorded *P. oryzicollum*, *P. heteroogonium, P. longipapillum* causing pre and post damping – off, seed Decay, crown rot, on rice (*Oryza sativa*) in Iran (Salmaninezhad and Mostowfizadeh-ghalamfarsa, 2019). In Iraq a very old study was conducted by Hassan (1979) to diagnoses the diseases caused by *Pythiaceae* on cucumber resulted in isolation of the *P. butleri* from the root. Recently, characterization of *Pythium* species related with several crops has greatly depended on sequences of the "internal transcribed region (ITS) of the ribosomal DNA (rDNA)" using ITS1 and ITS4 primers (Paul, 2001; Mathew, *et al.*, 2003). However, similar studies were missing for *Pythium* spp. in Iraq. Therefore, the objectives of this research were to isolate and identify other *Pythium* species addition to *P. aphanidermatum* in the greenhouse and to investigate the diversity of the *Pythium* spp. by study their morphological, molecular and pathoginicity characterization on Zucchini *Cucurbita pepo* in Baghdad and Al-anbar provinces in Iraq.

Materials and Methods

Collection of samples

Samples of soil and diseased zucchini & cucumber seedlings and fruit were collected from different greenhouse of cucurbits in several regions in Baghdad (Abu-ghraib, Jadiriyah, Taji, Madain, youssoufia, Mahmudiyah and Tarmia) and Al-anbar (Habbaniyah, Ramadi and Ssaklawia). The samples were collected separately into sterilize plastic bags and labeled and brought back immediately to the laboratory.

Isolation and identification of *Pythium* spp.

The plant samples were washed and cut into approximately 1 cm^2 pieces, surface sterilized in 1.50 % sodium hypochlorite (NaOCl), rinsed and blotted on sterilized paper. Plant pieces were then placed on Potato Dextrose Agar (PDA). Isolates from soil were obtained by plant traps methods using (zucchini, cucumber, beet, melon and corn seed) and (cucumber and apple fruits). Ten seeds or two fruits were sterilize and placed in the pot that filled with soil samples for three to ten days at glasshouse, all pots were moisture four times weekly. Then diseased seedling or fruit colonized by mycelium were collected and the pathogen was isolated from plants tissues as previously described.

For identification, the isolates were culture on corn meal agar (CMA) and potato carrot agar (PCA) at 25ºC for 7-14 days. Morphological features of Plaats – Niterink (shape of sporangia, Oogoia, antheridia, Oospores and appressoria figuration (Van der Plaats – Niterink, 1981) were examined under light microscope and recorded from all samples.

Effect of Media on Colony Growth

To determine the effect of different media type on isolates growth, four types of media were used: Potato-Dextrose Agar (PDA), Carrot Agar (CA), Corn Meal Agar (CMA) and V-8Juice Agar (V-8JA). The media were each centrally cultured with 5mm mycelial plug taken from growing margin of three-day old subcultures on 2% water agar (WA). The petri dishes were incubated at 25 ± 2°C. Diameters of Colony at right angles were measured after three-day incubation and the colony morphology was observed.

Effect of Temperature on Colony Growth

A 5mm mycelial plug from margins of three-day old cultures were placed on a PDA plate and incubated at temperatures of 10, 25, 30 and 35°C. Colony diameters at right angles were measured after three days incubation.

Pathogenicity test of isolates

The inoculums of isolates for the pathogenicity were prepared using *Sorghum bicolor* grains as described by (Al-maliky, *et al.,* 2018). Pathogenicity test of *pythium* spp. was conducted using soil mixture (sand and peat 2:1), and the mixture was sterilize with steam at 100 °C for 1h. The mixture was inoculated in plastic pots (15 cm- diameter) filled with the soil mixture and mixed with 2% W/W of inoculum. The pots were watered and covered with a plastic bag for two days. Ten zucchini seeds (Alexandria) were planted in each pot. The experiment was arranged in a complete randomized design (CRD) under glasshouse conditions with temperatures of 25 ± 4°C. Four replicate were prepared for each isolate. Disease incidence was record over a period of 30 days and the pathogen was re-isolate from seedlings and identified based on morphology as previously described.

DNA Extraction

The DNA was extracted from mycelium of *Pythium* spp. isolates were prepare by transfer four plugs from growing margin of three day old culture on PDA into flask containing 50ml of Potato Sugar broth (200g potato, 10g Sugar/ 1L water) (PSB). The flask was incubated at 30°C for seven days or until the mycelia cultures were

more than 3cm in diameter. Cultures were filtered through a layer of fine mesh cloth, washed three times with sterilized water. Two hundred mg of mycelium in liquid nitrogen was grounded to a fine powder using sterile mortar and pestle. The genomic DNA was extracted with manufacturer's procedure with the DNA extraction kit (Intron biotechnology/Korea). The extracted DNA was checked by gel electrophoresis through 1% agarose gel under 1×TBE electrophoresis buffer at 70V for 50 min.

PCR and Phylogenetic Analysis

The amplification of ribosomal DNA- Internal Transcribed Spacers (rDNA-ITS) region from 24 isolates of *Pythium* spp. were performed with universal primers pair ITS-1 (5'-TCCGTAGGTGAACCTGCGG- 3') as a forward primer and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') as a reverse primer (White *et al.,* 1990).

PCR was performed in 20µl reaction containing 2 µl PCR buffer 3 µl $MgCl_2$, 0.5 µl of dNTP Mix, 0.5 µl of ITS1 primer, 0.5 µl of ITS4 primer, 0.5 µl *Taq* DNA polymerase (Bioneer South korea) and 1µl of template DNA finally, 12µl of de ionized water was added. The amplification was carried out using PCR thermal cycler using 0.2µl tubes. The program cycle includes pre-heating duration at 94°C for 5min followed by 35 cycles of denaturing at 95°C for 30 seconds, annealing temperature at 60°C for 30 sec, extension temperature at 72°C for 45 sec and final extension at 72°C for 5 min fallow by 4°C. The PCR product was confirmed using 1.5% agarose gel under 1x electrophoresis TBE buffer. Gel was stained with Ethidium bromide, photographed using gel documentation system. The size of amplified DNA of ITS region was estimated by DNA Ladder SiZer™ -100 bp.

PCR products were sequencing for both directions using ITS1 and ITS4 primers by (DNA sequencer 3730XL, Applied Biosystem). Sequences for both strand were edited manually with Bio Edit Sequence Alignment Editor Software program (version 5.0.9) to obtain the completed ITS region. The results were analyzed for similarity using Basic Local Alignment Search Tool (BLAST) in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTN search (Altschul *et al.,* 1997) at the National Center for Biotechnology Information (NCBI), (http://www.ncbi.nlm.ngh.gov). All sequences obtained from ITS region were deposited in GenBank. Multiple sequence alignment was performed using CLUSTAL W program (http://www.ebi.ac.uk/Tools/services/web clustalw2) and edited manually using Bioedit program. The other sequences of the species were obtained from GenBank NCBI (http://www.ncbi.nlm.nih.gov) to generate phylogenetic tree. The sequences data were performed in MEGAX software to draw the phylogenetic tree (Tamura *et al.,* 2007). The evolutionary history relations were inferred based on the distance method by using the Neighbor-Joining method (Saitou, and Nei, 1987). The percentage of replicate trees in which the associated isolates were clustered together in the bootstrap test (500 replicates) was shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and were calculated in units as number of base substitutions per site.

Results and Discussion

Isolation and identification of *Pythium* spp.

A total of 24 *Pythium* spp. isolates were collected from 10 different regions in Baghdad and Al-anbar Table1. Fourteen isolates from the greenhouse in Baghdad region (Abu-ghraib, Jadiriyah, Taji, Madain, youssoufia, Mahmudiyah and Tarmia) and ten isolates from Al-Anbar regions (Habbaniyah, Ramadi and Ssaklawia). Two isolates (S2 and S3) were identified as, *Pythium deliense* Fig. 1 and the others 22 isolates were *P. aphanidermatum* Fig. 2 *P. deliense* was recovered from Ssaklawia only in Al-anbar. Depend on the number of *P. aphanidermatum,* species was commonly found in the most of cucurbits greenhouses in both of provinces in Iraq. Most of isolates grown on CMA were formed white, cottony, and fluffy with abundant aerial mycelium except of S2 & S3 isolates had a little loose aerial growth. The results of Morphological features show that S2 and S3 isolates were produced flamentous & inflated sporangia, Oogonia was smooth, intercalary or terminal, and the stalk of oogonia was curved, towards the antheridia and Aplerotic oospores. The others 22 isolates were produced oogonia with a straigth stalk. The Morphological characteristics of the isolates were the same with those described by van der Plaats-Niterink (1981).

In Iraq Al- Doory *et al.,* (1959) reported *P. aphanidermatum* (Edson) Fitz for the first time and reisolate from cucumber as causal agent of root rot by El-Behadle & Al-Azawi, 1979. This species has been widely studied in Iraq but, for our knowledge this is the first record of *P. aphanidermatum* as a pathogen agent of damping off on zucchini. Also this is the first report of *P. deliense* in Iraq.

Effect of Media on Colony Growth

The results showed that all the 24 isolates of *Pythium* spp. grew on all media types (V8-A, CA, CMA & PDA)

Table 1: Location & Types of sampling , isolate methods and isolates code.

Location	Sample Type/ Isolate method	Isolate code
Al-anbar / Habania	Diseased Plant/ Zucchini	H1
Al-anbar / Habania	Soil/ Plant Trap/cucumber seed	H2
Al-anbar / Habania	Soil/ Plant Trap/ melon seed	H3
Baghdad/ Abu-ghraib	Diseased Plant/ cucumber	A1
Baghdad/Abu-ghraib	Diseased Plant / cucumber	A2
Baghdad/Abu-ghraib	Diseased Plant / cucumber	A3
Baghdad/Abu-ghraib	Diseased Plant / cucumber	A4
Baghdad/Abu-ghraib	Diseased Plant / cucumber fruit	A5
Al-anbar/ Ssaklawia	Diseased Plant / Zucchini	S1
Al-anbar/ Ssaklawia	Diseased Plant / Zucchini	S2
Al-anbar/ Ssaklawia	Diseased Plant / Zucchini	S3
Al-anbar/ Ssaklawia	Soil/Plant Trap/ cucumber fruit	S4
Al-anbar/ Ssaklawia	Soil/Plant Trap/ Corn seed	S5
Al-anbar/ Ssaklawia	Soil/Plant Trap/ zucchini seed	S6
Baghdad/ Jadiriyah	Soil/Plant Trap/ beet seed	J1
Baghdad/ Jadiriyah	Soil/Plant Trap/ Apple fruit	J2
Al-anbar/ Ramadi	Diseased Plant / cucumber	R1
Baghdad/ Taji	Soil/Plant Trap/ beet seed	T1
Baghdad/ Tarmia	Soil/Plant Trap/ cucumber fruit	Tr1
Baghdad/ Mahmudiyah	Soil/Plant Trap/ cucumber fruit	Ma1
Baghdad/ Taji	Soil/Plant Trap/ Corn seed	T2
Baghdad/Youssoufia	Diseased Plant / Zucchini	Y1
Baghdad/ Madain	Diseased Plant / cucumber	M1
Baghdad/ Madain	Diseased Plant / cucumber	M2

within three day. The averages of growth rates were between 6.3-8.6 cm on V8-A, 6-7.3cm on CMA and 5.96-7.15 cm on PDA while the lowest growth rates 5.8-6.5cm on CA. However, V8-A and CMA supported the best growth followed by PDA. Significant differences (P=0.05) in growth rates were report among isolates on the same media. Most of isolate were grow faster than S4 & S5 on V8-A. on CMA, S2& S3 were record the highest growth rates 7.4 & 7.2 respectively.

Effect of Temperature on Colony Growth

The mycelium growths of *Pythium* spp. were significantly affected by the temperature. All the isolate were grow faster at 30°C attaining rates of 8-8.4cm followed by 5.96-7.15 cm at 25°C. For all isolates, growth limited at 10°C. Therefore, ecologically, the pathogens are well suited to grow and multiply in the greenhouse of cucurbits growing areas in Baghdad and Al-anbar /Iraq. This results are agree with those reported by van der plaats-Niterink (1981) revered that *P. aphanidermatum* and *P. deliense* were grow and distribute in the warm regions and the optimum temperatures were 30-40°C. In Iran Teymoori founded that these two species had the same optimum temperatures (Teymoori, *et al.,* 2012).

Pathogenicity test of isolates

The results of the pathogenicity tests of 24 isolates of *Pythium* spp. on Zucchinin plant in the greenhouse are shown in (Table 2). No symptoms appeared in control treatment and. Mycelia of pathogens were re-isolated from infected seedlings. All the isolates were record significant differences in the Disease Incidence (pre and post emergence (% damping- off). *P. aphanidermatum* isolates (H1, H2 & H3) were report the highest percentages of pre emergence reach (52.00,44.00 &43.00)% respectively. For the other isolates, the pre-emergence of damping off were range between (42.00-10.00)%. The isolates of *P. deliense* S2 & S3 were show pre-emergence of damping off reach (43.00 & 37.00)% and post each (37.00 & 31.00)% respectively. Results show highly variations and differences on the pathogenicity of all isolates in both provinces on zucchini plant. There variations could be a potential ability to affect many cucurbits crops that planted on those regions. These differences may be because may have deferent ability of releasing the analysis plant tissues enzymes (Raju *et al.,* 2002; Lee *et al.,* 2010)., Pathogenicity of *P. deliense* is proven for the first time on this study.

PCR and Phylogenetic Analysis

Amplification of the ITS region yielded PCR product fragment length of 823-889 bp using primers ITS1 and ITS4 (Fig. 3).

The ITS sequences of all *Pythium* spp. isolates were deposited in the NCBI GeneBank database. The accession numbers (MN365079 - MN365100), (MN365124 & MN365125) and the consensus sequences of strands are presented in Table 3.

BLAST analyses of the NCBI GeneBank database with the ITS sequences of the culture isolates from soil and diseased plants recovered ITS sequences of *P. aphanidermatum and P. deliense* (Table 4). The isolates S2 & S3 were report the highest ITS-rDNA-scoring matches were with *P. deliense* AY598674 (100%) from Canada. Other 22 isolates were identified as *P. aphanidermatum* and showed maximum identity rang (98.99-100) when compared with the recovered ITS sequences from GeneBank of KU211462, EU162761 (USA), KJ696535 (China), DQ298521 (Oman), KX228070 (Iran), MH393376 (Egypt), KP274886 (Turkey) and AY598622 (Canada).

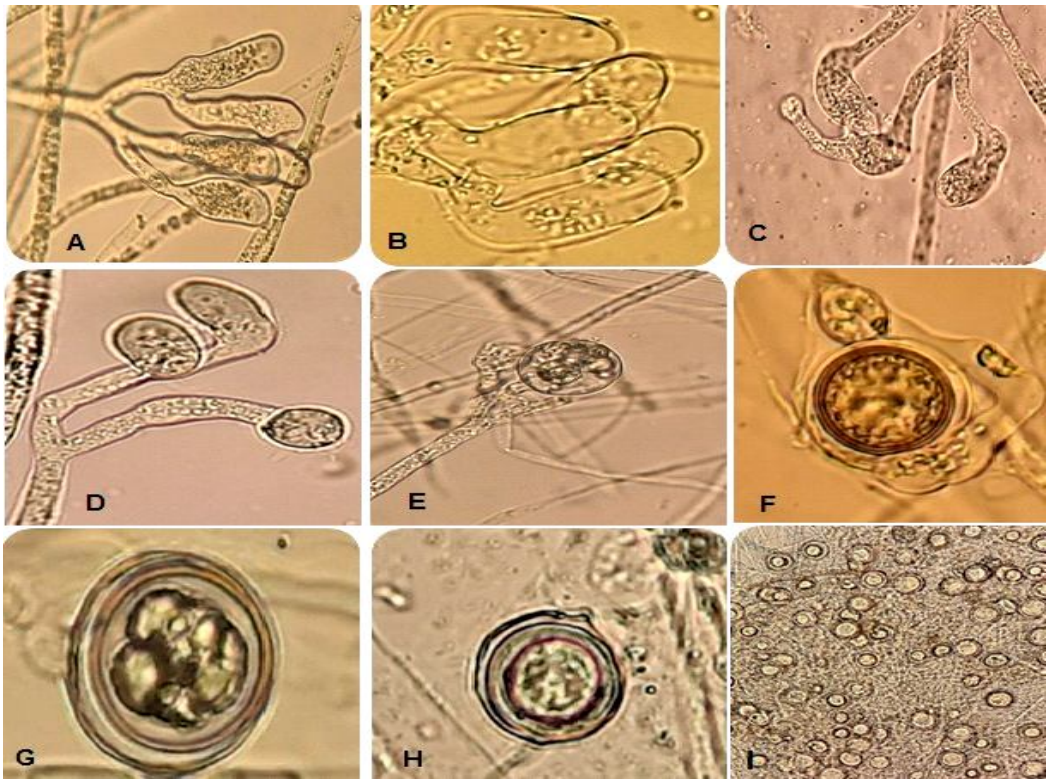

Fig. 1: Morphological characteristics of *Pythium aphanidermatum* : (A&B) Sporangia (C & D) appressaria (E) contact between oogonium & antheridium (F) formation of oospores (G-I) Aplerotic oospores.

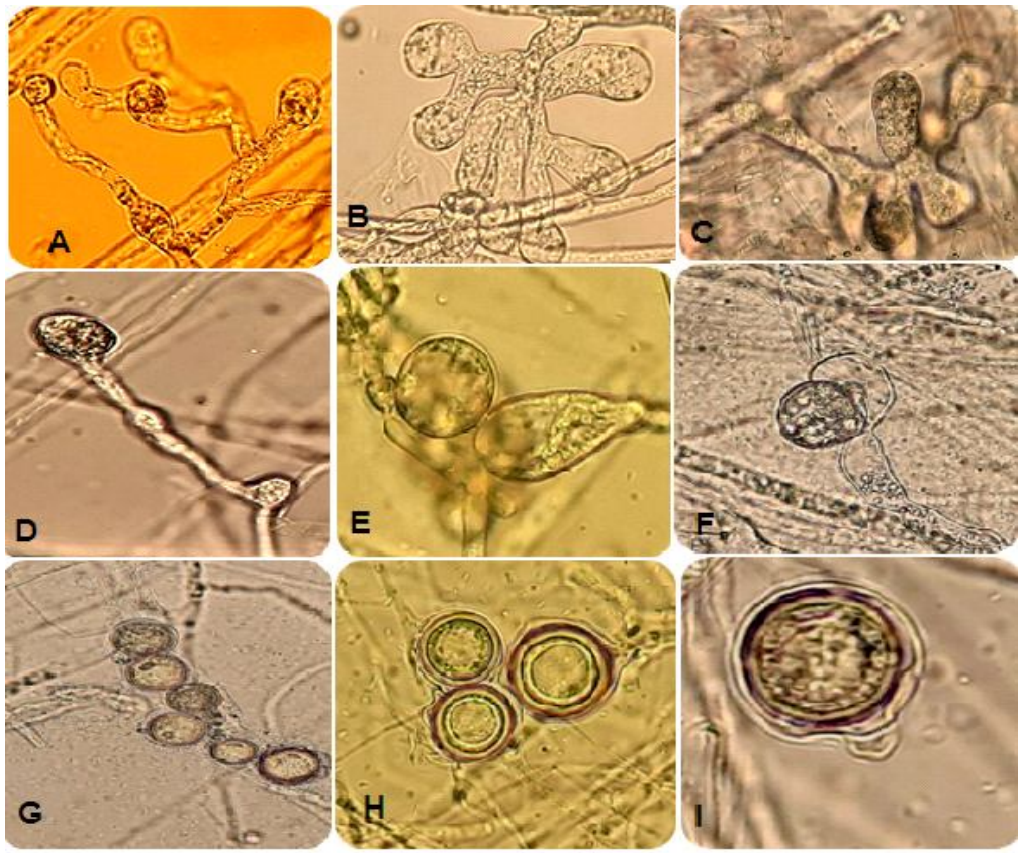

Fig. 2: Morphological characteristics of *Pythium deliense* : (A & D) appressaria (B & C) Sporangia (E & F) contact between oogonium & antheridium (G-I) Aplerotic oospores.

Table 2: Pathogenicity test results of Pythium spp. isolates on Zucchini showing disease incidence percentage after 4 weeks incubation.

Isolate	Disease Incidence (% damping off)	
	Pre-emergence*	Post-emergence*
H1	52.00	41.00
H2	43.00	38.00
H3	44.00	39.00
A1	19.67	15.00
A2	39.00	29.00
A3	38.00	31.00
A4	35.00	40.00
A5	36.00	23.00
S1	42.00	25.00
S2	43.00	37.00
S3	37.00	31.00
S4	27.00	20.00
S5	23.00	19.00
S6	33.00	32.00
J1	36.00	29.00
J2	18.00	22.67
R1	20.33	27.00
T1	29.00	32.00
Tr1	30.00	29.00
Ma1	22.00	12.00
T2	35.00	15.00
Y1	14.00	14.00
M1	10.00	11.00
M2	14.00	12.00
Control	0.00	0.00
L.S.D	6.14	6.26

* Each value is the mean of four replicates.

Table 3: The GeneBank accession numbers and the consensus sequences of the 24 *Pythium* spp. isolates from ITS region.

Isolate code	GeneBank	Consensus
	Accession No.	sequence(bp)
H1	MN365124	847
H2	MN365079	859
H3	MN365080	865
A1	MN365081	825
A2	MN365082	824
A3	MN365084	889
A4	MN365125	863
A5	MN365098	855
S1	MN365083	823
S2	MN365089	827
S3	MN365090	854
S4	MN365093	854
S5	MN365099	864
S6	MN365100	851
J1	MN365085	857
J2	MN365086	825
R1	MN365087	865
T1	MN365088	867
Tr1	MN365091	855
Ma1	MN365092	857
T2	MN365094	864
Y1	MN365095	865
M1	MN365096	857
M2	MN365097	858

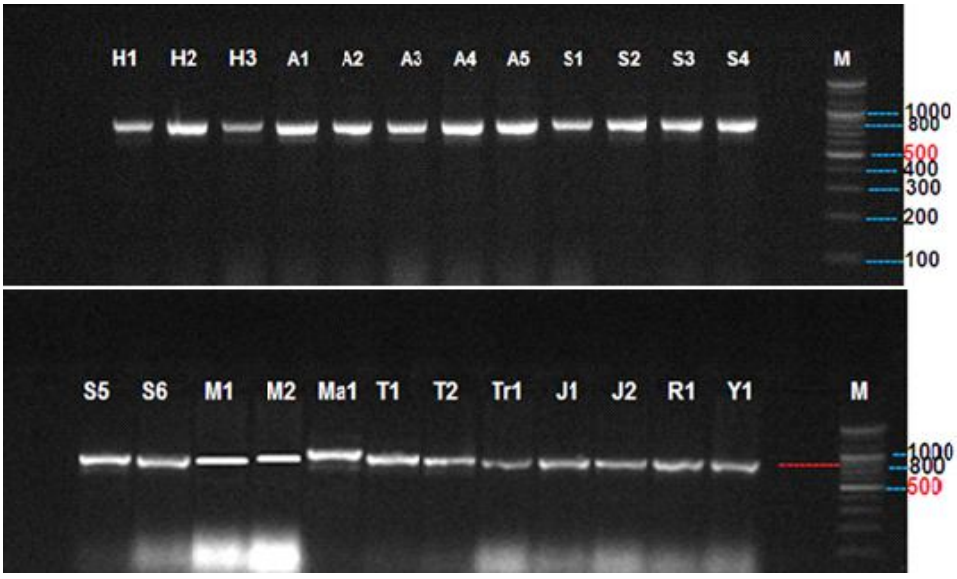

Fig. 3: Gel electrophoresis showing representative band of PCR product from ITS region for 24 isolates of Pythium spp.

Table 4: BLAST results of 24 *Pythium* spp. isolates for ITS region.

Isolate name	Similar to accession No.	Maximum identity%	Species
H1	KU211462	99.41	*P. aphanidermatum*
H2	KU211462	99.41	*P. aphanidermatum*
H3	KU211462	98.49	*P. aphanidermatum*
A1	MH393376	100	*P. aphanidermatum*
A2	MH393376	100	*P. aphanidermatum*
A3	AY598622	100	*P. aphanidermatum*
A4	KU211462	98.49	*P. aphanidermatum*
A5	MH393376	98.52	*P. aphanidermatum*
S1	KU211462	100	*P. aphanidermatum*
S2	AY598674	100	P. deliense
S3	AY598674	100	P. deliense
S4	EU162761	99.06	*P. aphanidermatum*
S5	KJ696535	99.53	*P. aphanidermatum*
S6	KU211462	100	*P. aphanidermatum*
J1	AM055944	99.98	*P. aphanidermatum*
J2	MH393376	99.88	*P. aphanidermatum*
R1	KJ755088	99.41	*P. aphanidermatum*
T1	KU211462	99.07	*P. aphanidermatum*
Tr1	DQ298521	99.56	*P. aphanidermatum*
Ma1	AY598622	99.30	*P. aphanidermatum*
T2	KU211462	99.07	*P. aphanidermatum*
Y1	KU211462	99.07	*P. aphanidermatum*
M1	AY598622	99.30	*P. aphanidermatum*
M2	MH393376	99.63	*P. aphanidermatum*

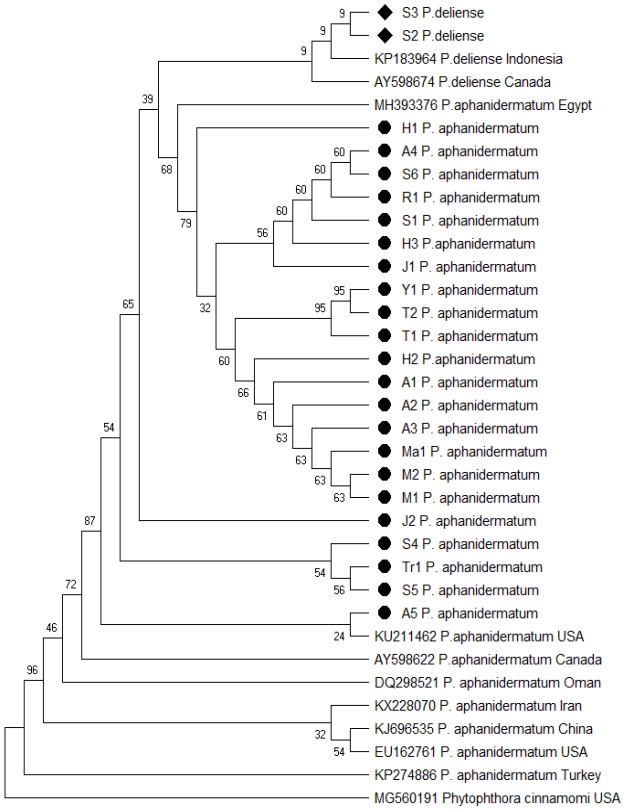

Fig. 4: Phylogenetic tree based on ITS region derived from neighbor-joining method showing the genetic relationships among 24 isolates of *Pythium aphanidermatum and P. deliense* and other species from GeneBank.

Table 5: Species, GenBank accession numbers and additional information of specimens used in the phylogenetic analyses of rDNA- ITS.

Selected species	GenBank accession No.	Country	Sequences length (bp)	Reference
P. aphanidermatum	MH393376	Egypt	851	Ahmed, *et al.*, 2018
P. deliense	AY598674	Canada	827	Levesque, *et al.*, 2004
P. deliense	KP183964	Indonesia	959	Panca, *et al.*, 2015
P. aphanidermatum	AY598622	Canada	851	Levesque, *et al.*, 2004
P. aphanidermatum	KJ696535	China	869	Li, *et al.*, 2014
P. aphanidermatum	EU162761	USA	847	Olson, *et al.*,2016
P. aphanidermatum	KU211462	USA	880	Rojas, *et al.*, 2017
P. aphanidermatum	KX228070	Iran	777	Salmaninezhad, *et al.*, 2017
P. aphanidermatum	DQ298521	Oman	777	Al-sa'di, *et al.*, 2007
P. aphanidermatum	KP274886	Turkey	842	Lehtijärvi, *et al.*, 2014
Phytophthora cinnamomi	MG560191	USA	827	Weiland, *et al.*, 2018

For phylogenetic analysis, sequences of ITS region (ITS1-5.8S-ITS2) were used after omitting the suspicious sequences at the ends of the regions. The phylogenetic tree of *P. aphanidermatum and P. deliense* isolates in this study and other reference species (11) from GeneBank (Table 5) were designed based on the distance method (neighbor-joining) (Fig. 4). *Phytophthora cinnamomi* was used as outgroup.

P. deliense formed distinct clade from isolates of *P. ahpanidermatum* and it was grouped with the isolates from GeneBank from Canada and Indonesia. The Iraqi isolates of *P. ahpanidermatum* were well separated from

the other from (USA, Canada, China, Iran, Oman and Turkey. this clade was divided in to seven subcalde of isolates. Most of Iraqi isolates form this study was more closely to that isolate from Egypt. Also isolate A5 was grouped with KU2111462 from USA.

Isolates from Iraq were show highly variations also they were deferent from GeneBank isolates. These differences may be because of the geographically or because of the Pathoginicity.

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