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Characterization of *Pythium aphanidermatum* Isolated from Diseased Cucumber Plants in Jordan

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Abstract: The pathogen of *Pythium aphanidermatum* is a very important pathogen causing disease and yield losses on vegetables. Correct identification of pathogens lead to correct and effective control strategies and methods. However, the traditional methods based on morphological and physiological identification becomes difficult and time consuming because of the similarity amongst the different groups of species and intraspecific morphological variation frequently observed in different field isolates. Molecular techniques based on several DNA methods have been developed to identify *Pythium* spp. and also to understand the relationships between species. Morphology and physiology characteristics of *Pythium aphanidermatum* were investigated for identification and variability. The optimum pH levels of *Pythium aphanidermatum* grow *in vitro* were 7 and the optimum growing temperatures of the isolates recovery in this study was 30°C. The mycelial were thick and white with fluffy topography. Each isolate produced aseptate, hyaline mycelium, the oogonia terminal, globose and smooth. Antheridia mostly intercalary, sometimes, broadly sac shaped, monoclinous or diclinous, thick walled aplerotic oospores and lobed sporangia. Sequences of selected isolates obtained in this study were matched *Pythium aphanidermatum* (KY646468) from GenBank. The UPGMA dendrogram showed that there were variations within the population of *Pythium aphanidermatum* at similarity coefficient 0.1. These isolates obtained from diseased cucumber plants collected from different farms in the Jordan Valley in April 2017.

Key words: *Pythium aphanidermatum*, cucumber, root rot, sequence of ITS1, Jordan Valley, UPGMA dendrogram

INTRODUCTION

The genus *Pythium* was created by Pringsheim in 1858 with the description of *Pythium monospermum* Pringsh as the type species. More than 200 species have been described worldwide (Mathew *et al*., 2003; Abdelghani *et al*., 2004) but only 120 species have been given names (Dick, 1990) and the rest were placed in five groups (F, T, G, P and HS) (Van der Plaats-Niterink, 1981; Dick, 1990). Members of the genus *Pythium* are now classified as fungal-like organisms in the Phylum Oomycota in the Kingdom Chromista and not in the Kingdom Fungi. The fungal-like organisms have mycelium lacking septa and both molecular and biochemical studies suggest that they are closer to algae (Abdelghani *et al*., 2004) than true fungi. However, these organisms infect plants in similar ways to true fungi (Agrios, 2005). The Phylum Oomycota contains some of the most economically important plant pathogens such as those belonging to the genera *Pythium*, *Phytophthora* and *Aphanomyces* (Deacon, 2006).

The main and traditional methods for identifying *Pythium* species are based on morphological and physiological studies (Van der Plaats-Niterink, 1981). The main features used for morphological identification are sporangial size and shape, production of zoospores, formation and morphological features of oospores, the number and shape of antheridia and the way in which antheridia attach to oogonia (Van der Plaats-Niterink, 1981; Dick, 1990; Singleton *et al*., 1993). However, morphological identification becomes difficult and time consuming because of the similarity in morphological features amongst the different groups of species and intraspecific morphological variation frequently observed in different field isolates (Van Os, 2003). Incorrect identification could lead to control strategies and methods that are ineffective.The main physiological features used for identification of *Pythium* species are those based on cardinal temperatures (minimum, optimum and maximum) (Van der Plaats-Niterink, 1981).

More recently, molecular techniques based on several DNA methods have been developed to identify *Pythium* spp. and also to understand the relationships between species (Bruns *et al*. 1991; Levesque *et al*. 1998; Levesque and De Cock, 2004; Al-sheikh, 2010). Most of these methods involve amplifying a fragment from the Internal Transcribed Spacer (ITS) region of rDNA by Polymerase Chain Reaction (PCR). This is a highly

variable region and has a large number of characters that can be used to identify organisms, including Oomycetes, to the species level (Matsumoto *et al.* 1999; Paul and Masih, 2000; Paul, 2001; Mathew *et al.* 2003).

Sequence analysis of the ITS region of rDNA has been used to identify and study the relationships among isolates of *Pythium* species (Paul, 2000; Bailey *et al.*, 2002; Mathew *et al.*, 2003; Schurko *et al.*, 2003; Levesque and De Cock, 2004; Paul 2006; Al-Sheikh, 2010). Scott *et al.* (2005) sequenced the ITS 1, 5.8S rDNA and ITS 2 for three groups of *Pythium* isolates obtained from table beet in the LockyerValley in Queensland and compared these sequences to the databases in GenBank. They recorded that group Lockyer Valley *Pythium* A (LVP A) was identical to the corresponding sequence from *P. aphanidermatum*, group LVP B was identical to the corresponding sequence from *P. ultimum* and group LVP C was identical to the corresponding sequence from *P. dissotocum*.

Pythium spp. are very important pathogens causing disease and yield losses in vegetables (Agrios, 2005; Deacon, 2006) with the main diseases being seed rot, seedling damping-off and root rot. *Pythium* spp. can be found in most crops around the world including cucumber (Stanghellini *et al.*, 1988; Cherif and Belanger, 1992). Tesoriero *et al.* (1991) reported that *Pythium aphanidermatum* was found on cucumber and capsicum crops grown in soilless systems in New South Wales (NSW) Australia and resulting in yield losses.

In Jordan, there is no study characterized *Pythium aphanidermatum*. One study has been investigated the disease control caused by *Pythium aphanidermatum* (Al-Ameiri, 2014). This study is designed to characterize *Pythium aphanidermatum* recovery from diseased cucumber plants grown in Jordan Valley based on morphological and physiological features and molecular methods using sequences of ITS region of rDNA.

MATERIALS AND METHODS

Recovery of *Pythium* cultures: *Pythium* species were isolated from roots of diseased cucumber grown in Jordan Valley at April 2017. *Pythium* isolates were obtained from three different farms in different areas. Root systems were washed under tap water and root pieces were plated on Potato Dextrose Agar (PDA). Petri dishes were incubated in the dark at 25±1°C for 2 days (Van der Plaats-Niterink, 1981). Cultures were identified as *Pythium aphanidermatum* by morphological examination using a light microscope.

Morphological studies: The morphological characters of representative isolates of *Pythium aphanidermatum* isolates including hyphal diameters, oogonia, oospores and oospore wall thicknesses at 400x total magnification using an eyepiece micrometer. Other features studied were: the number, shape and arrangement of antheridia; The size and shape of oogonial projections (spines) and the shape and abundance of appressoria forming at the points of contact with the petri dish. The *Pythium aphanidermatum* cultures were 5 days old grown on PDA.

Physiological studies

Effect of pH levels: Four isolates were selected to study the effect of pH on growth *in vitro* for *Pythium aphanidermatum* and examined on PDA. The pH of the medium was adjusted to various levels 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 by adding 0.1 N sodium hydroxide and 0.1 N hydrochloric acid. Five mm discs taken from 5 days old culture were inoculated and incubated under 12 h light and 12 h dark at 25±1°C for 5 days. Three replications were used for each isolate and treatment. The diameter of the colony growth was measured and recorded after 5 days.

Effect of temperature: Eight temperatures from 5-40°C (5°C intervals) were used to incubate PDA Petri dish cultures of the four *Pythium aphanidermatum* isolates selected to find out the optimum temperature as well as, the lowest and the highest temperatures at which growth occurred. All incubation was carried out under 12 h light and 12 h darkfor 5 days. Three replicates were used for each isolate at each temperature. Growth was measured after 5 days, using two diameter measurements perpendicular to each other. General Linear Model (GLM) ANOVA was used to differences (p 0.05) between treatments mean (SPSS Ver. 10).

Molecular methods: Mycelium of *Pythium aphanidermatum* isolates were used to extract the DNA and PCR was used to amplified the Internal Transcribed Spacer (ITS1) region of the ribosomal nuclear DNA (rnDNA) follow the same methods of Paul (2000). The sequencing of ITS1 was carried out and maintained at a commercial facility (Macrogen Inc., Seoul, South Korea) following the stander methods. BLAST search (http://blast.ncbi.nlm.nih.gov) was used to analyze the sequences and ClustalW (http://www.ebi.ac.uk) was drown the phylogram of the isolate and its relative.

RESULTS AND DISCUSSION

Isolation: All isolates recovery from diseased cucumberroots collected from Jordan Valley area were identified as *Pythium aphanidermatum* based on the morphological characteristics according to Pythiumkey (Van der Plaats-Niterink, 1981).

Morphological characterization: The morphology characterization of the selective four isolates of *Pythium aphanidermatum* recovery from Jordan Valley area, indicated that the growth on the PDA produced a thick, white cottony mycelial growth with fluffy topography. Each isolate produced aseptate, hyaline mycelium (3.4 μm to and double 6.2 μm), the oogonia terminal, globose and smooth 20-25 μm diameter. Antheridia mostly intercalary, sometimes, broadly sac shaped, 10-14 μm long and 10-14 μm wide, 2 per oogonium, monoclinous or diclinous, thick walled aplerotic oospores (17-9 μm) and lobed sporangia.

Physiological studies

Effect of pH levels: The optimum pH level of the four isolates of *Pythium aphanidermatum* tested was 7. Moreover, all isolate tested grew very well at pH levels of 6. At pH levels of 5 and 8, all isolates tested were grew slightly well. However, slightly growth was appeared at pH levels of 3, 4 and 9 for all isolates tested (Table 1).

There were no significant differences in colony growth between isolates tested at pH 6 and 7 for all *Pythium aphanidermatum* isolates tested (p = 0.56). However, no significant differences found between the colony growth at different pH levels for each isolate (p = 0.00) (Table 1).

Effect of temperature: Isolates of *Pythium aphanidermatum* have an optimum growth temperature of 30°C. They also grew very well at temperature of 25 and 35°C. At temperature 20°C *Pythium aphanidermatum* grew well (28.60). Limited growth was occurred at temperatures of 10 and 40°C but no growth was appeared at temperature of 5 and 45°C (Table 2).

Table 1: Mean growth (mm) of 4 isolates of *Pythium aphanidermatum* incubated on PDA with nine pH levels (3-9) in the dark. Growth was measured after 3 days. Within a column, means followed by the same letter are not significantly different from each other at p≤0.01. n = 3 for each isolate at each temperature

	Mycelial growth of different isolates (cm^2)			
pH level	Isolate 1	Isolate 2	Isolate 3	Isolate 4
3	5.93[a]	5.38[a]	4.97[a]	5.02[a]
4	10.41[b]	13.64[b]	12.75[b]	11.89[b]
5	33.66[c]	31.65[c]	31.92[c]	32.65[c]
6	56.72[d]	55.06[d]	56.72[d]	55.11[d]
7	56.72[d]	56.72[d]	56.72[d]	56.72[d]
8	20.75[e]	19.35[e]	20.07[e]	18.89[e]
9	3.79[f]	3.52[f]	2.78[f]	3.94[f]

[a-f]Significant values

There were significant differences in colony growth between the four *Pythium aphanidermatum* isolates tested at each temperature (p = 0.01). Moreover, there were significant differences between the colony growth at different temperatures for each isolate (p = 0.00). Nevertheless, there were no significant differences in colony growth for each isolate between temperatures of 25 and 35°C (Table 2).

Sequence of ITS region of rDNA: Sequences of the selective four isolates obtained in this study matched *Pythium aphanidermatum* (KY646468) from GenBank. The sequence of isolate N1-N3 were 790-794 bp in length and were 100% identical to the corresponding sequence from *Pythium aphanidermatum* (KY646468). Moreover, the sequence of isolates N4-N6 were 749-780 bp in length were 100% identical to the corresponding sequence from *Pythium aphanidermatum* (KY646468). However, the sequence of isolate N7-N9 were 647-660 bp in length were 99% identical to the corresponding sequence from *Pythium aphanidermatum* (KY646468). Furthermore, isolates N10-N12 were 600-660 bp in length were 99% identical to the corresponding sequence from *Pythium aphanidermatum* (KY646468) (Table 3).

Variation observed using the sequence of ITS region of rDNA: Data described by sequence length (bp) of ITS region of rDNA were used to construct an UPGMA dendrogram (Fig. 1). The UPGMA dendrogram showed that there were variations within the population of *Pythium aphanidermatum* at similarity coefficient 0.1.

Table 2: Mycelial growth (cm^2) of 4 isolates of *Pythium aphanidermatum* incubated at nine different temperatures from 5-45°C on PDA in the dark. Growth was measured after 3 days. Within a column, means followed by the same letter are not significantly different from each other at p≤0.01. n = 3 for each isolate at each temperature

	Mycelial growth of different isolates (cm^2)			
Temperature (°C)	Isolate 1	Isolate 2	Isolate 3	Isolate 4
5	0.00[a]	0.00[a]	0.00[a]	0.00[a]
10	7.86[b]	7.45[b]	6.88[b]	7.32[b]
15	12.38[c]	11.34[c]	11.78[c]	22.05[c]
20	28.98[d]	27.87[d]	28.98[d]	28.56[d]
25	46.45[f]	43.65[f]	46.32[f]	44.87[f]
30	56.72[e]	56.72[e]	56.72[e]	56.72[e]
35	45.67[f]	44.87[f]	45.06[f]	44.95[f]
40	8.05[b]	8.21[b]	8.29[b]	7.98[b]
45	0.00[a]	0.00[a]	0.00[a]	0.00[a]

[a-f]Significant values

Table 3: Sequence length (bp) of ITS region of rDNA for 12 isolates of *Pythium aphanidermatum* from diseased tomato plants and comparison with sequences in GenBank.

Isolate code	Sequence length (bp)	Match from GenBank (Location)	GenBank accession number	Identities (%)	Gaps
N 1-3	790-794	*Pythium aphanidermatum* 2017 (Maryland)	KY646468	100	0
N 4-6	749-780	*Pythium aphanidermatum* 2017 (Maryland)	KY646468	100	0
N 7-9	647-660	*Pythium aphanidermatum* 2017 (Maryland)	KY646468	99	1
N 10-12	600- 660	*Pythium aphanidermatum* 2017 (Maryland)	KY646468	99	1

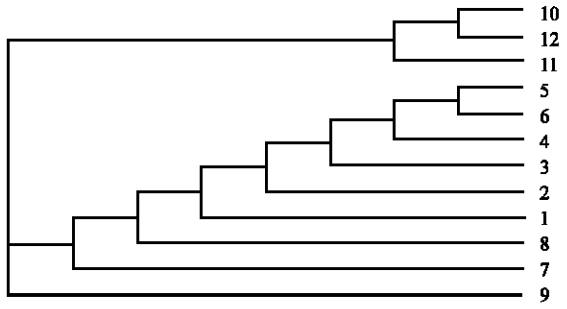

Fig. 1: Dendrogram based on sequence of ITS region of rDNA of the *Pythium aphanidermatum*

These isolates obtained from diseased cucumber plants collected from different farms in the Jordan Valley in April 2017. The isolates obtained from the same area showed the same morphological and physiological characters were grouped together in the UPGMA dendrogram (Fig. 1).

The research investigating the pathogen of *Pythium aphanidermatum* isolated from diseased roots of cucumber from Jordan Valley is the first study to characterize the pathogen based on morphology and molecular methods. In Jordan, there was a single study on *Pythium aphanidermatum* on disease management was done using *Trichoderma harzianum* to reduce cucumber damping-off disease under field conditions (Al-Ameiri, 2014). However, there were few studies on *Pythium aphanidermatum* on the west bank (Palestinian) were done on disease control and pathogen ecology (Ali-Shtayeh and Saleh, 1999; Ali-Shtayeh *et al*., 2003). Moreover, this study found that there were variation in the population on *Pythium aphanidermatum* isolated from cucumber roots from Jordan Valley. This variation effect pathogen management methods and need more research by using different methods in order to keep the disease under control.

Pythium aphanidermatum isolates recovery from diseased cucumber roots collected from Jordan Valley area in this study were identified based on the morphological characteristics according to *Pythium* key (Van der Plaats-Niterink, 1981). However, morphology features sometime are similar within thesame species and sometimes lead to incorrect identification (Van Os, 2003). These differences may be appear by the effect of environmental factors (such as, temperature) and physiological factors (such as nutrition) (Agrios, 2005). Morphological characteristics, however are still useful and often provide the basis for species identification. Moreover, sequence of ITS1 region were used to confirm the identification by using BLAST search in the GenBank.

Four isolates were selected to represent all recovery isolates in this study for the morphology characterization of *Pythium aphanidermatum* recovery from Jordan Valley area, indicated that the growth on the PDA produced a thick, white cottony mycelial growth with fluffy topography. All isolates produced aseptate, hyaline mycelium (3.4 μm to and double 6.2 μm), the oogonia terminal, globose and smooth 20-25 μm diameter. Antheridia mostly intercalary, sometimes, broadly sac shaped, 10-14 μm long and 10-14 μm wide, 2 per oogonium, monoclinous or diclinous, thick walled aplerotic oospores (17-9 μm) and lobed sporangia. These results are contestant with the results describe in the *Pythium* key (Van der Plaats-Niterink, 1981). Moreover,the results found in the present study are consist with the result describe by Ashwathi *et al.* (2017) who characterize *Pythium aphanidermatum* morphologically *in vitro* isolated from several coriander growing regions of Tamil Nadu, India.

The physiological studies for the selective isolates of *Pythium aphanidermatum* show that the optimum growth temperature is 30°C. Moreover, the isolates grew very well at temperature of 25 and 35°C. However, limited growth was occurred at temperatures of 10 and 40°C but no growth was appeared at temperature of 5 and 45°C. The results of the present study found the optimum pH level of the selective isolates of *Pythium aphanidermatum* tested was 7. Moreover, all isolate tested grew very well at pH levels of 6. At pH levels of 5 and 8, all isolates tested were grew slightly well. However, very limited growth was appeared at pH levels of 3, 4 and 9 for all isolates tested. *Pythium aphanidermatum* is well known as a high temperature pathogen and do more damage to plants in worm weather (Agrios, 2005). A study on soybean found the temperature of 24-36°C is the best temperature of *Pythium aphanidermatum* to affect their roots and do significant damage (Thomson *et al*., 1971; Al-Sheikh, 2010). Jordan Valley area has a worm and humidity weather most of the year which mean suitable for the pathogen of *Pythium aphanidermatum*.

The sequences of the ITS region of rDNA for the twelve *Pythium aphanidermatum* isolates, selected to represent isolates obtained to confirm the identification to species level. The twelve isolates were found to be identical to *Pythium aphanidermatum* (KY646468) in the GenBank. Sequences of the ITS region of rDNA gives a correct identification in most resent researches (Pavon, *et al*., 2010; Ozkilinc *et al*., 2018).

CONCLUSION

This study demonstrated that there were variations in the population of *Pythium aphanidermatum* recovery from cucumberroots grown in Jordan Valley based on the sequences of the ITS region of rDNA. This variation could be related to different farms that use different

agriculture techniques and the environmental conditions in the area of Jordan Valley which is high temperatures and humidity. Molecular techniques have been used to investigate the variation between populations of one species obtained from different areas or different environmental condition or even different host (Sukmawati and Miarsyah, 2017). The method used in the present study, sequences of the ITS region of rDNA, more accurate to develop a clear vision of the variation in the population of plant pathogens. Several researches reported that the sequences of the ITS region of rDNA is the best technique to drew a map of the population of plant pathogen (Ozkilinc *et al*., 2018).

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