

Morphological Characterization and Pathogenicity of *Pythium* sp. Infecting Cucumber Seedlings in Greenhouses

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ABSTRACT

Greenhouse-grown cucumber seedlings exhibiting typical symptoms of *Pythium* damping-off and root rot were collected. The causal agents were isolated on *Pythium* selective medium; nystatin, ampicillin, rifampicin, and miconazole (NARM) in potato dextrose agar (PDA). Four different isolates were isolated and their pathogenicity was tested using cotyledon and young seedlings assays. Results of these isolates showed variation in their ability to infect cucumber seedling. Isolate "GZ-11" was highly pathogenic, caused 100 % pre- and post-emergence damping-off. The isolate was identified to the species level based on morphological characters along with sequences of the internal transcribed spacer (ITS) of the ribosomal DNA and showed 99% similarity to *Pythium spinosum* (Synonym of *Globisporangium spinosum*). Aggressiveness of *P. spinosum* was investigated at two levels of inoculum concentrations (10 and 20 g/kg soil). Results reported that *P. spinosum* was highly aggressive and significantly reduced cucumber seed germination compared to the control.

Keywords: *Pythium*, pathogenicity, cucumber, ITS and phylogenetic tree

INTRODUCTION

Cucumbers (*Cucumis sativus*) are one of the most economically important crops worldwide and can be grown under greenhouse and field condition (Davis et al. 2008). The total production of cucumber in Egypt 2018 was 457,795 tones (FAO, 2018). Cucumbers are affected by several diseases such as root rot (Chatterton and Punja, 2009), *Fusarium* wilt (Zhao et al. 2012), *Pythium* damping-off, *Rhizoctonia* damping-off (Kazerooni et al, 2019), downy mildew and powdery mildew (Wang et al. 2008). Damping-off and root rot are one of the most common disease affecting cucumber production.

Pythium is the major causal organism of this disease, especially under high humidity and cold conditions (McCarty and Miller, 2002). Most species of them have a wide range of hosts but some are restricted to certain family members causing reduction in crop yield.

Pythium attacks embryo, hypocotyl, and emerging radicle causing pre- and post-emergence damping-off disease. Roots of mature plant are also infected limiting plant growth and sometimes causing death (Schroeder et al. 2013). Shoot and root dry weights of cucumber plant were significantly decreased by *Pythium ultimum* infection (Ravnskov et al. 2020). Several *Pythium* species can infect cucumber seedling including *Pythium aphanidermatum*, *P. ultimum*, *P. deliense*, *P. myriotylum*, and *P. spinosum* (Paul et al. 1996; Wulff et al. 1998; Roberts et al. 2007; Al-Balushi et al. 2018).

Identification of the *Pythium* species is based on the growth rate and morphological features on specific media, such as shape of sporangia, oogonia, and antheridia (Van der Plaats-Niterink, 1981). This requires high experience and a new techniques have been developed to facilitate and more accurate identification. Recently, several molecular tools including Species-specific molecular primers, Polymerase Chain Reaction (PCR) and the sequence of the ras-related protein gene have been developed (Moorman et al. 2002; Godfrey et al. 2003; Klemsdal et al. 2008)

The goal of this study was (i) to isolate and identify *Pythium* spp. associated with cucumber damping-off based on morphological features and molecular characterize. (ii) Screening pathogenicity of the *Pythium* isolate and (iii) Study the influence of inoculum density on disease severity on cucumber seedling.

MATERIALS AND METHODS

Sample collection and *Pythium* isolation

Infected cucumber seedlings were collected from greenhouses and open fields (Menoufia and Giza government, January 2017). The samples were washed under running tap water to remove soil debris and sterilized sequentially with 3% (v/v) sodium hypochlorite (NaOCl) for 3-5 min, 70% (v/v) ethanol for 1 min and then transferred to sterile distilled water for 3 min. The crown and root were cut into fragments (0.5-1 cm long each) and placed on potato dextrose agar (PDA) plates supplemented with nystatin (10 µg/ml),

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ampicillin (200 µg/ml), rifampicin (10 µg/ml) and miconazole (1 µg/ml) as an antibacterial (Morita and Tojo, 2007). The plates were incubated at 25°C till pathogen growth was observed on the samples. *Pythium* spp. were morphologically identified according to the typical morphological features using light microscopy (GIPPON, GLCD-120, JAPAN) and hyphal tipped to obtain pure cultures.

Pathogenicity test

Petri dish test: Pre-emergence damping-off was tested in petri plates. Cucumber seeds (HAYEL, Hybrid cucumber) were surface sterilized as mentioned before. Seeds were germinated on moist, sterile filter paper for 2 days before being used. Ten seeds were placed in a 9 cm petri plate containing 1% (w/v) water agar medium. For each *Pythium* isolate, one plug was inoculated to each plate and incubated at 25 °C for seven days. Seeds were placed on non-inoculated plate as a control.

Pots test: All *Pythium* isolates were tested for pathogenicity (Post-emergence) on cucumber seedling at the 2-true leaf stage as described by Feng and Dernoeden (1999) with some modification. Plastic cups of 7 cm diameter, with drainage holes, were filled with autoclaved 2:1 (v/v) sand-peat mixture and one seedling was transplanted per cup. After two days, two mycelium plugs (7 mm in diameter) taken from the edge of 5-days old culture were placed around the base of each seedling and covered with a thin layer of soil. Agar plugs were applied to control cups. Seedlings were incubated at 25°C in growth chamber with 12h/day of fluorescent light and irrigated every 2 days with sterile distilled water. For each treatment, there were four randomly arranged replicates. The percentages of post-emergence damping-off were recorded for two weeks after transplanting.

Effect of *Pythium* density on disease severity

Pythium inoculum was prepared by growing three plugs on sterilized corn-sand medium, which contained corn seeds, sand and deionized water at the ratio 1:1:2 (V/V/V) respectively, for 10 days at 25°C. The inoculum was then mixed with sterilized clay soil at a rate of 10 and 20 g/kg soil. Plastic pots (500-ml) were filled with the infested soil and planted with five surface-sterilized seeds. Negative control was established by planting seeds in non-infested soil. Pots were watered manually to maintain soil moisture. Three replicates were prepared for each treatment and the experiment was repeated twice. Pre-damping off was calculated, after three weeks of planting, by comparing the emergence percentage with that in control.

Statistical analysis

All experiments were carried out twice in triplicate and the obtained data were analyzed by analysis of variance (ANOVA) using the Wasp version 2.0. The Critical Difference (CD) at P = 0.05 was used to compare the means controls against the rest of the treatments.

Oomycetes identification using Internal transcribed spacer (ITS) region

Genomic DNA was extracted according to CTAB method as described by Doyle *et al.*, (1987). The ITS region was amplified using primers ITS1 (5'-TCT GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The reaction mixture (25µl) contained 5µl of Taq red buffer, 20 ng of template DNA, 10 pmol of each primers, 0.25 U of Taq polymerase (Bioline, UK). PCR cycling conditions were: 95°C for 2 min, 30 cycles of 95°C for 1 min; 50°C for 1 min; 72°C for 1 min; 1 cycle of 72°C for 5 min. The PCR product was checked on 1.5% agarose gel stained with 0.05 µg/ml ethidium bromide and the target band was purified using the GF-1 PCR clean-up kit (Vivantis Technologies, Sdn. Bhd.) according to the manufacturer's instructions.

Sequence analysis

The purified PCR product was sequenced and data obtained were compared with those in GenBank database of National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the BLAST algorithm. Phylogenetic analysis was performed using MEGA version X version 10.0.5 (<https://www.megasoftware.net/>).

RESULTS AND DISCUSSION

Pathogenicity of *Pythium* spp.

Four *Pythium* spp. were isolated from the diseased plant during winter 2017. The tested isolates showed a wide range in their pathogenicity (Table 1). Two isolates (MF-7 and MF-15) were either nonpathogenic or have low level of pathogenicity to cucumber seedling. The isolate MF-22 was moderately pathogenic and cause pre- and post-emergent damping of at the rate 79% and 50% respectively. Among the tested isolates, GZ-11 isolate was found to be the highest pathogenic, causing seed rot and seedling damping-off at rate 100%, and selected to be identified. Wei *et al.* (2011) in study with eight *Pythium* spp reported that *Pythium ultimum* was the most pathogenic species resulting in 97% seed rot and 46.4% damping-off on soybean. Rossman *et al* (2017) observed variations in the virulence of the *Pythium* isolates in causing seed rot and pre-emergence damping-off of dry bean.

Table 1. Effect of *Pythium* isolates on seed germination and seedling healthy. Significant differences ($p < 0.05$) between means were followed by different letters

Isolate code	Location	Seed germination (%)	Healthy seedling (%)
MF-7	Menoufia	100 ^a	75 ^a
MF-15	Menoufia	100 ^a	100 ^a
GZ-11	Giza	0.0 ^c	0.0 ^b
MF-22	Menoufia	76.6 ^b	50 ^{ab}
CD ($P = 0.05$)		8.04	0.588
CV (%)		6.12	67.89

Morphological and identification of *Pythium* sp.

Morphological characterization of the selected isolate (GZ-11) was similar to *Pythium spinosum* (Figure 1). Colonies grew rapidly, daily growth rate 30-35 mm, on PDA medium at 25°C with white, dense aerial mycelium. Main hyphae was 2-5 µm wide (av. 3.7 µm) with occasionally varying numbers of blunt,

finger-like projections. Sporangia and zoospore not formed. Oogonia were globose or fusiform, intercalary or terminal, 15-24 µm (av. 19 µm) diameter, mostly smooth but occasionally with blunt protuberances. Antheridia were typically monoclinal, 1 per oogonium. Oospore plerotic, 15-22 µm (av. 18 µm) diameters, thin-walled (av. 1.3 µm).

Molecular characterization of *Pythium spinosum*

Genomic DNA of *Pythium* isolate was extracted and the ITS rDNA region (ITS1-5.8S-ITS2) was amplified by PCR (Figure 2). After visualization of the PCR product on 1.5% agarose gel, a specific DNA band was shown at the length of 1000 bp. The oomycetes identification was confirmed by sequence comparison of the ITS region with sequences obtained from the Nucleotide Database of (NCBI). The sequence of *Pythium* isolate (GZ-11) was closely related with that of *Globisporangium spinosum* MK910133 (Basionym: *Pythium spinosum*) with 99% similarity (Figure 3).

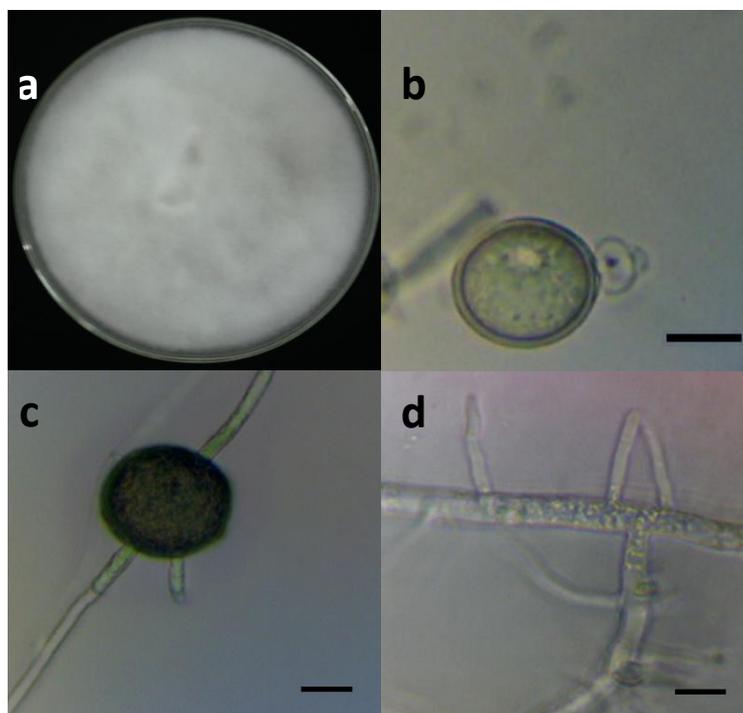


Fig. 1. Morphology of *Pythium spinosum*. (a) Colony patterns at 25 °C on PDA, (b) Monoclinous antheridium attached to a oogonium, (c) intercalary oogonium with a projection, (d) hyphae with spine-like projections. Scale bars = 10 µm

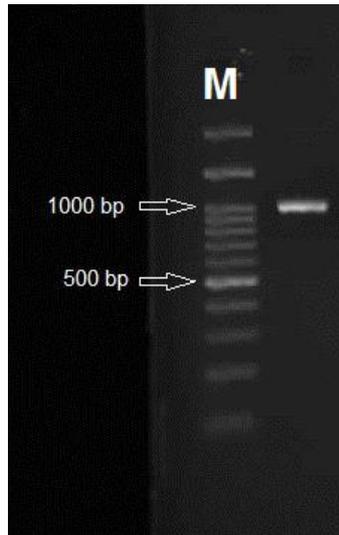


Fig. 2. Electrophoresis of the PCR product of ITS rDNA of *Pythium spinosum* on 1.5% agarose gel (lane M: 1.5 Kb DNA Marker)

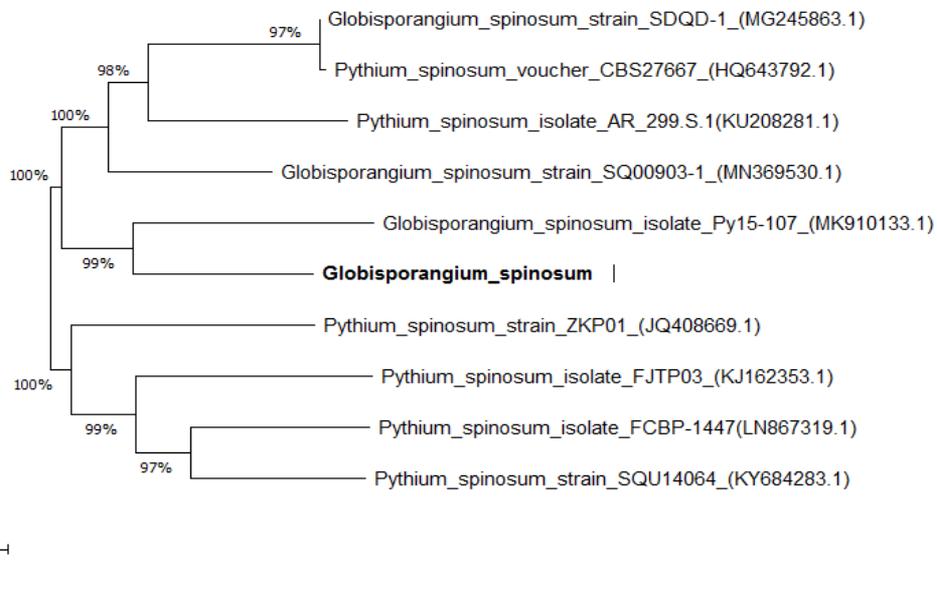


Fig. 3. Unrooted Phylogenetic tree showing the position of *P. spinosum* isolate based on internal transcribed spacer (ITS) sequence of nuclear rDNA

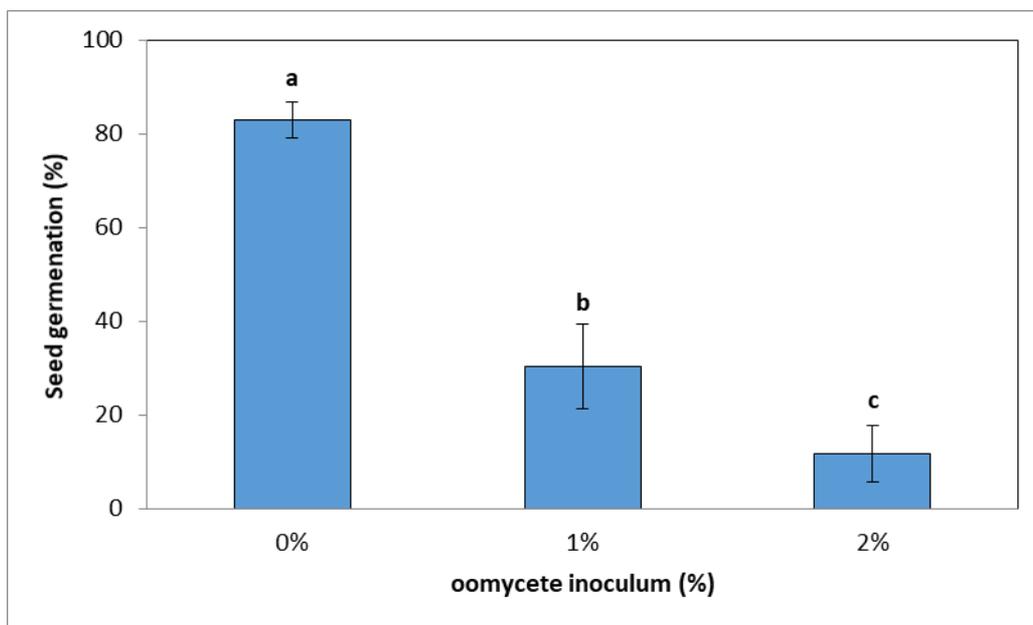


Fig. 4. Effects of *Pythium spinosum* inoculum concentrations on cucumber seed germination. Vertical bar represent standard error

Influence of inoculum density on severity of damping-off disease

The seed germination percentage was significantly ($p < 0.001$) influenced by the inoculum concentrations in the soil (Figure 4). Within three weeks, *P. spinosum* isolate was found to exhibit high level of aggressiveness, even at inoculum densities as small as 10 g/kg soil, as compared to the control.

The result also showed that disease severity was directly proportional to the oomycete inoculum size. Our results were similar to Hwang *et al.* (2001) who found that the survival of pea seedling decrease as inoculum concentration of *P. irregulare* and *P. ultimum* increased. Development of *Pythium* Stunt disease on lettuce was enhanced by increasing soil inoculum density of *P. tracheiphilum* (Sauvageau *et al.* 2019).

Conflict of interest

The author(s) they do not have any conflict of interest

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الملخص العربي

الخصائص المورفولوجية والقدرة الإمراضية للبيثيوم يصيب بادرات الخيار في الصوب الزراعية

محمد مدحت غريب, جابر أبو زيد, شيماء بشير و السيد السيد حافظ

خصائصها المورفولوجية بالإضافة إلى التحليل التسلسلي لمنطقة ITS في الحمض النووي الريبوزي والتي أظهرت تشابهاً بنسبة 99% مع بيثيوم سبينوسيوم. تم التحقق من القدرة الإمراضية للبيثيوم باستخدام كثافات مختلفة من اللقاح (10 و 20 جم/كجم تربة) في التربة المزروعة. أفادت النتائج قدرة إمراضية شديدة لعزله البيثيوم سبينوسيوم حيث انخفضت نسبة إنبات بذور الخيار بشكل معنوي مقارنة بالشاهد غير المعامل.

جمعت عينات لنباتات خيار تظهر عليها أعراض مرض موت البادرات وعفن الجذور من داخل صوب زراعية، حيث تم عزل المسبب المرضي على وسط إنتخابي للبيثيوم يتكون من بيئة PDA مضاف إليها نيساتين وأمبيلين و ريفامبيسين و ميكونازول (NARM). عُرِزت أربعة أنواع مختلفة من البيثيوم وأختبرت قدرتها الإمراضية على بادرات لنبات الخيار (هجين هايل). أظهرت العزلات تبايناً في قدرتها على الإصابة. تسببت العزلة GZ-11 في إحداث أعلى نسبة إصابة 100% للبادرات قبل وبعد الإنبات. عُرِفَت العزلة بالإعتماد علي