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ORIGINAL ARTICLE

First report of *Pythium aphanidermatum* infecting tomato in Egypt and its control using biogenic silver nanoparticles

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Abstract

In August 2016, tomato plants grown during a hot, wet summer with heavy soil flooding, displaying symptoms of wilting, dead plant, root rot with crown and stem rot, at Beni Suef and Fayoum governorates were examined. A number of 16 fungal isolates were isolated from tomato plants displaying the above symptoms. These isolates were classified as belonging to six species, namely: *Alternaria solani*, *Chaetomium globosum*, *Fusarium solani*, *Fusarium oxysporum*, *Pythium* spp. and *Rhizoctonia solani*. Isolates of *Pythium* spp. were prevalent and were found to be more pathogenic than the other fungal isolates. This species causes damping-off, root rot, sudden death, stem rot and fruit rot. The pathogen was identified as *Pythium aphanidermatum* based on morphological, cultural, and molecular characteristics. Biogenic silver nanoparticles (AgNPs) were produced using the *F. oxysporum* strain and characterized by transmission electron microscopy (TEM). The size of these spherical particles ranged from 10 to 30 nm. *In vitro*, biogenic AgNPs showed antifungal activity against *P. aphanidermatum*. In greenhouse and field experiments, AgNPs treatment significantly reduced the incidence of dead tomato plants due to root rot caused by *P. aphanidermatum* compared to the control. All of the investigated treatments were effective and the treatment of root dipping plus soil drenching was the most effective. To the best of our knowledge, this study describes *P. aphanidermatum* on tomato in Egypt for the first time. Also, biogenic AgNPs could be used for controlling root rot disease caused by this pathogen.

Key words: biogenic synthesis, *Pythium aphanidermatum*, silver nanoparticles (AgNps), tomato

Introduction

Tomato (*Lycopersicon esculentum* L.) is considered to be a major vegetable crop grown worldwide. In Egypt, tomato cultivation covers about 32% of the total vegetable-growing area; with total production being approximately 16% of total vegetable production (Bekheit and Latif 2015). In Egypt, several soil-borne plant pathogens such as *Rhizoctonia solani*, *Fusarium solani*, *Phytophthora capsici*, *Sclerotium rolfsii* and *Fusarium oxysporum* are reported to attack tomato plants, causing damping-off and root rot diseases (Ghonim 1999; Saad 2006; Morsy *et al.* 2009; Mohamed *et al.* 2015; Hamza *et al.* 2016; Shenashen *et al.* 2017). There is little information available about the possibility of *Pythium* spp. inciting damping-off and root rot diseases on tomato in Egypt. The *Pythium* species are fungal-like organisms and are generally known as damping-off fungal pathogens (West *et al.* 2003). Among these, *Pythium aphanidermatum* (Edson) Fitzpatrick is a major causal agent of damping-off, root rot and wilt of tomato all over the world (Jayaraj *et al.* 2005; Christy Jeyaseelan

et al. 2012; Kipngeno *et al.* 2015). Also, Le *et al.* (2003) found that *P. aphanidermatum* plays a key role in sudden death disease and causes severe damage to tomato plants grown under field conditions following flooding at high soil temperatures. Diverse disease management strategies including synthetic chemical fungicides have been used for the control of the diseases caused by *P. aphanidermatum* (Shafique *et al.* 2016). However, their broad host range, rapid germination of sporangia and formation of oospores make it difficult to control such diseases (Nelson 1987). Research to find alternatives to pesticides has been persistently conducted because the overuse of pesticides causes ecological and environmental problems and has harmful effects on human beings. Among many natural compounds, silver nanoparticles have been used to manage fungal plant pathogens (Park *et al.* 2006; Jo *et al.* 2009; Min *et al.* 2009; Rai *et al.* 2009). In recent years, extensive research has been done on the biosynthesis of silver nanoparticles based on bacteria, fungi, and plant extracts due to their eco-friendly protocol and better morphological control (Narayanan and Sakthivel 2010; Buhroo *et al.* 2017). For example, silver nanoparticles were produced extracellularly by the fungus *F. oxysporum* (Ahmad *et al.* 2002). The aim of our study was to isolate, identify and test the pathogenicity of pathogen(s) associated with tomato plants displaying symptoms of wilting, dead plant, root rot with crown and stem rot. Biological synthesis of silver nanoparticles (AgNPs) as well as their activity in controlling *P. aphanidermatum* under *in vitro*, greenhouse and field conditions were also investigated.

Materials and Methods

Tomato sample collection

In August 2016, tomato plants grown during a hot, wet summer with heavy soil flooding, displaying symptoms of wilting, dead plant, root rot with crown and stem rot, at Beni Suef and Fayoum governorates were examined. Samples of damaged tomato plants displaying the above symptoms were collected from local, open fields located in both governorates. The root and foliage systems of diseased tomato plants were separated, using a sterilized scalpel, and both organs transported to the laboratory in sterilized plastic bags.

Isolation and identification of the causal pathogens

The infected tomato roots and stems, separately, were gently washed under running tap water, cut into small pieces, about 0.5–1.0 cm in length, superficially sterilized by sodium hypochlorite solution (5%) for 2 min, washed several times with sterile distilled water and dried between sterilized filter paper (Elshahawy *et al.* 2016). The small, superficially sterilized pieces were then placed on the surface of potato dextrose agar (PDA) plates. After 5 days of incubation at 25°C (±2°C), the frequency of occurrence (%) of grown fungal colonies was recorded. Single spores or hyphal tip techniques were used for fungal purification, and each colony was cultivated on other PDA plates for 5 days at 25°C (±2°C) to verify purity. All fungal isolates recovered were then transferred to PDA slants for maintenance till use in subsequent experiments. Tentative identification of isolates was carried out on the basis of their morphological and cultural characteristics (Gilman 1957; Booth 1971; Barnett and Hunter 1972; van der Plaats Niterink 1981; Sneh *et al.* 1991).

Pathogenicity tests

Damping-off incidence

Potting soil (loamy clay) was placed in cloth bags (500 g each) and sterilized by autoclaving at 1.5 atmos for 1 h, three times successively, then left in the laboratory for 4 days to be well aerated. The contents of the bags were put in sterilized plastic pots (30 cm diameter); the soil filled 2/3 of the pots. Sterilization of pots was carried out by dipping them in 5% formaldehyde solution for 15 min, then left to dry for 2 weeks to get rid of formaldehyde before use. The pots containing soil were transferred to a greenhouse and divided into 17 lots (each lot consisted of 4 pots); one was left un-inoculated (to serve as control) while the other 16 lots were infested with the fungal isolates to be tested: *Pythium* sp. (P1), *Pythium* sp. (P2), *Pythium* sp. (P3), *F. solani* (Fs1), *F. solani* (Fs2), *F. solani* (Fs3), *F. solani* (Fs4), *F. oxysporum* (Fo1), *F. oxysporum* (Fo2), *F. oxysporum* (Fo3), *R. solani* (Rs1), *R. solani* (Rs2), *R. solani* (Rs3), *Alternaria solani* (As1), *Chaetomum globosum* (Cg1) and *Ch. globosum* (Cg2).

Inocula were prepared by growing each fungal isolate in 250 ml Erlenmeyer flasks containing 100 g of barley-sand medium (1 : 1 plus 40% distilled water) using agar discs (0.5 cm diameter) taken from the hyphal tips of 7-day-old culture grown on PDA plates for 10 days at 25°C (±2°C). Infestation was carried out by thoroughly mixing the fungal cultures with the soil of each pot at a ratio of 4% of soil weight. The pots were watered every 2 days to achieve homogenous distribution of fungal inocula in the soil, and were left for 2 weeks before planting.

Apparently healthy seeds of tomato cv. Castle rock were used and treated with 2% sodium hypochlorite solution for 1 min, washed thoroughly in a series of sterilized water. Ten surface sterilized seeds were sown, at equal distances, in each pot. The pots of each lot were placed on an assigned bench in the greenhouse, and then maintained at 28°C (±2°C) and 70% relative

humidity (RH) for 30 days. The pots were examined daily and irrigated as needed to maintain adequate moisture for plant growth and disease development.

The experimental period was 30 days after sowing. During the experimental period, the germination percentage was observed every 7 days after sowing. The incidence of post-emergence damping-off disease was recorded during the experimental period based on the symptoms noticed on diseased plants up to 30 days after sowing (Florence 2011). Average seedling biomass was also taken 30 days after sowing.

Root rot incidence

Sterilized plastic pots (30 cm diameter) were filled with 5 kg autoclaved loamy clay soil artificially infested individually with each of the tested fungal isolates at a ratio of 4% of soil weight. Two weeks later, healthy tomato seedlings (30-day-old, cv. Castle rock) were transplanted into plastic pots at the rate of two seedlings/pot. Four experimental units were used for each fungal isolate as well as control treatment (non-infested soil). An experimental unit consisted of five pots. Disease assessment was conducted daily for 30 days and was initiated 1 day following planting. During the experimental period, disease incidence (the percentages of dead plants due to root rot) was recorded (Abd-El-Khair *et al.* 2016). Average plant biomass was also determined 30 days after transplantation.

Sudden death incidence

Sterilized plastic pots (30 cm diameter) were filled with 5 kg autoclaved loamy clay soil. Healthy tomato seedlings (30-day-old, cv. Castle rock) were transplanted into plastic pots at the rate of two seedlings/pot. Six weeks later, inocula of each of the tested fungal isolates were added to the top of pots at a ratio of 10 g/pot and the pots were flooded immediately for 48 h after inoculation (Le *et al.* 2003). Four experimental units were used for each fungal isolate as well as control treatment (non-infested soil). An experimental unit consisted of five pots. The pots were maintained at 30°C (±2°C). Disease incidence as the number of wilted plants 4 and 7 days after soil flooding was recorded.

Stem rot incidence

Sterilized plastic pots (30 cm diameter) were filled with 5 kg autoclaved loamy clay soil. Healthy tomato seedlings (30-day-old, cv. Castle rock) were sown in plastic pots at the rate of two seedlings/pot. Six weeks later, to determine pathogenicity 16 fungal isolates were inoculated into two different sites of the stem (Morsy and Elshahawy 2016). Individual plugs from agar cultures of fungal isolates were placed on the stems of 10 seedlings. Ten inoculated plants with culture free agar plugs served as controls. Disease assessment was recorded daily for 10 days after inoculation.

Fruit rot incidence

Pathogenicity to tomato fruit was confirmed using 16 fungal isolates recovered from tomato samples. Pathogenicity tests were conducted using apparently healthy tomato fruits of Castle rock cultivar after they had been surface sterilized by soaking in 3% sodium hypochlorite solution for 3 min and washed thoroughly three times with sterile distilled water. Each fungal isolate was inoculated into ten tomato fruits. Surface sterilized tomato fruits were inoculated with PDA colonized by each of the fungal isolates individually (Elshahawy *et al.* 2017). A set of 10 fruits, which were inoculated with sterile PDA, served as control. The inoculated fruits were then kept in sealed plastic boxes and incubated at 25° (±2°C). Three days later, symptomatic fruits were observed.

Identification of *Pythium* spp.

Morphological and cultural characteristics

Preliminary identification of the three *Pythium* spp. isolates was done using morphological characters of mycelium and spores based on the keys of van der Plaats-Niterink (1981).

Molecular identification

The three isolates of *Pythium* sp. (P1, P2 and P3) were sub-cultured on vegetable juice medium (V8). Each isolate was inoculated in 25 ml of V8 medium (V8 juice 10%; $CaCO_3$ 0.1% and Agar 2%), then incubated in a shaker incubator at 28° (±2°C) at 120 rpm for 10 days. After the incubation period, the fungal mycelium from the three isolates were harvested and dried on absorbent paper. DNA was extracted from mycelium using CTAB protocol (Benito *et al.* 1993). The DNA of the different isolates was amplified by polymerase chain reaction (PCR) using ITS 4 and DC 6 set of primers. Forward primer DC 6 (5′-GAGGGACTTTTGGGTAATCA-3′) and the reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were designed based on sequences of 18S rDNA from a number of fungi (White *et al.* 1990; Bonants *et al.* 1997). The final 50 µl reaction mixture contained 1× PCR buffer (NEB, England), 1 nmol of dNTPs, 1 pmol of 2 mM $MgSO_4$, 0.25 pmol of forward and reverse primers, 1 unit Taq DNA polymerase (NEB, England) and 10 µl template DNA (Barakat *et al.* 2017). The PCR program started by an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 60 s and final extension at 72°C for 10 min. The amplified products were separated on 1% agarose gels in 1X TBE (Tris-borate-EDTA) buffer. Gels were photographed under UV light (Kheiralla *et al.* 2016). The PCR products were purified by QIAquick Gel Extraction Kit (QIAGEN, USA) and run on agarose gel to achieve the

purification of 18S rDNA fragments for sequencing. The identification was achieved by comparing the contiguous 18S rDNA sequence with the 18S rDNA sequence data from the reference and type strains available in public databases GenBank using the BLAST program (National Centre for Biotechnology Information) (http://www.ncbi.nlm.nih.Gov/BLAST/). The sequences were aligned using Jukes Cantor Model. The sequences of these strains were recorded in GenBank and the accession numbers were obtained (Barakat *et al.* 2015).

Fungal biosynthesis of silver nanoparticles (AgNPs)

The *F. oxysporum* strain was obtained from the Agricultural Microbiology Department, National Research Centre, Egypt. This strain was used for the biosynthesis of silver nanoparticles from silver nitrate salt. *Fusarium oxysporum* was activated by streaking on a PDA plate. After activation, one disc was inoculated into 250 ml flasks containing 100 ml sterilized potato dextrose broth (PDB) medium and incubated in a rotary shaker (120 rpm) at 28°C (±2°C). The biomass after 3 days of incubation was harvested by filter paper (Whatman No. 1). The supernatant was used to convert silver nitrate to silver nanoparticles (El-Naggar *et al.* 2014). Ten ml of supernatant was added to 10 ml of 1 M silver nitrate solution in bottles with caps. The bottles were incubated under static conditions at room temperature for 24 h. The samples were collected and analysed to detect the production of silver nanoparticles.

Characterization of silver nanoparticles (AgNPs) using UV/VIS spectrophotometer detection

Silver nanoparticles were detected by scanning the collected samples at ranges between 200 and 800 nm using UV/VIS spectrophotometer (Jenway UV/Visible-2605 spectrophotometer, England).

Characterization of silver nanoparticles (AgNPs) using transmission electron microscopy (TEM)

The formation of AgNPs was investigated by high-resolution transmission electron microscopy (HRTEM) JEOL (JEM-2100 TEM). The size and shape of these nanoparticles were studied.

In vitro evaluation of antifungal activity of biosynthesized silver nanoparticles (AgNPs)

Linear growth assays

The antifungal effect of AgNPs was examined against the linear growth of *P. aphanidermatum* (P2) *in vitro* using the pouring plate method (Min *et al.* 2009). Potato dextrose agar medium (1 l; pH 6.5–6.8) containing 200 g potato, 20 g dextrose and 20 g agar was prepared and 99 ml were placed in 250 ml conical flasks and then autoclaved. Before solidification, 1 ml of stock solution containing AgNPs was poured into the growth media prior to plating in five Petri dishes (90 mm in diameter) to obtain the desired concentrations of 40, 80, 120, 160 and 200 mg · l^{-1}. After 48 h of incubation, agar plugs of uniform size (5 mm diameter) containing cultures of *P. aphanidermatum* (P2) were inoculated simultaneously at the center of each Petri dish containing AgNPs, followed by incubation at 28°C (±2°C) for 7 days. Another set of AgNPs-free Petri dishes were used as control. Ten Petri dishes were used as replicates for each treatment as well as the control treatment. After 7 days of incubation, average colony radius and percentages of growth reduction were measured. The percentages of fungal growth reduction were determined according to the following formula:

$$\text{Fungal growth reduction \%} = (C - T)/C \times 100,$$

where: C – the diameter of mycelial growth i=n control plates, T – the diameter of mycelial growth in treated plates.

Zoospore germination assays

The inhibitor effect of AgNPs was examined against the zoospore of *P. aphanidermatum* (P2) *in vitro* using the zoospore germination assay method (Min *et al.* 2009). The protocol for the production of zoospores was adapted from Chae *et al.* (2006). Equal volumes of the zoospores and AgNPs treatments (at the same concentrations as in the previous experiment) were mixed. These mixtures were kept at room temperature for 24 h and samples were observed under a light microscope to count the percentage of non-germinated zoospores. A minimum of 50 zoospores was counted per treatment.

In vivo evaluation of antifungal efficacy of biosynthesized silver nanoparticles (AgNPs)

Greenhouse experiments

To assay the controlling activity of AgNPs on *Pythium* root rot of tomato in soil artificially infested with *P. aphanidermatum* (P2), the present experiment was carried out under greenhouse conditions. Based on the results obtained from *in vitro* studies, AgNPs at the concentration of 200 mg · l^{-1} and tomato seedlings cv. Castle rock were used. The experiments were conducted with a completely randomized design with four treatments (root dipping in AgNPs, soil drenching with AgNPs, root dipping plus soil drenching with AgNPs and infected control), each with four experimental units. An experimental unit consisted of five sterilized plastic pots (25 cm diameter) each containing

5 kg of pre-autoclaved infested loamy clay with *P. aphanidermatum* (P2) as previously described in the pathogenicity test. For the root dipping treatment, tomato seedlings at the 4–5 true leaf stage were gently uprooted and the roots were washed under running tap water to remove the potting mixture. After draining excessive water, seedling roots were dipped for 30 min in AgNPs with 1% carboxymethyle cellulose (CMC), as the adhesive, and planted directly. For the soil drenched treatment, AgNPs were applied to potting soil at the rate of 200 ml/pot and planted directly. For the control treatment, distilled water was used in both root dipping and soil drenching treatments. In all cases, tomato seedlings were planted after 7 days of potting soil infestation and two seedlings/pot were transplanted The minimum and the maximum temperatures were 20°C (±5°C) and 30°C (±3°C), respectively. The plants were irrigated when necessary. The experimental period was 30 days after transplanting. During the experimental period, disease incidence (percentages of dead plants due to root rot) was recorded. At the end of the experiment, some vegetative growth parameters i.e. average plant height (cm), average number of leaves/plant and average plant biomass (g), were also estimated for the tomato plants which survived.

Field experiments

Naturally infested soil with *P. aphanidermatum* located in Beni Suef governorate, was chosen to estimate the efficiency of AgNPs in reducing *Pythium* root rot of tomato. The experiment was conducted during the summer of 2017. Three treatment methods designed under greenhouse conditions i.e. root dipping, soil drenching and root dipping plus soil drenching were used. For soil drenching, AgNPs were applied to the soil at the rate of 10 ml per hill and planted directly. Silver nanoparticles at the concentration of 200 mg · l^{-1}, and tomato seedlings cv. Castle rock were used. The experiments were conducted with a completely randomized design with four treatments (root dipping in AgNPs, soil drenching with AgNPs, root dipping plus soil drenching with AgNPs and non-treated control), each with four experimental units. An experimental unit consisted of three rows. The dimensions of each row were 6 m in length, 30 cm in height and 50 cm in width. Two tomato seedlings/hill were sown with 50 cm between hills. Irrigation and fertilization were conducted as generally recommended for tomato production regimes. An experimental period was 60 days after transplanting. During the experimental period, disease incidence (the percentages of dead plants due to root rot) was recorded. Some vegetative growth parameters viz. average plant height (cm) and average number of branches/plant were also estimated for the surviving plants.

Statistical analysis

Data were subjected to SPSS software version 14.0 and analyzed statistically by the analysis of variance test (ANOVA) and the different means were compared by Duncan's multiple range test at $p < 0.05$. Percent data were statistically analyzed after arcsine square-root transformation; however, untransformed data are presented.

Results

Symptomatology and frequency of fungal isolates associated with diseased tomato plants

Tomato plants grown in fields located in Beni Suef and Fayoum governorates in Egypt, developed symptoms

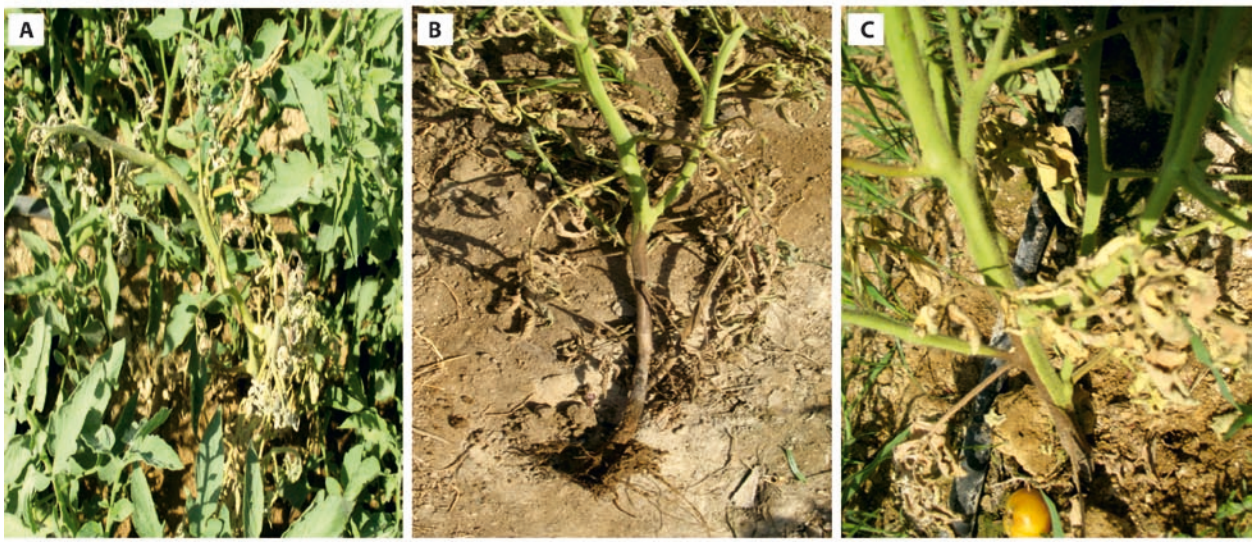

Fig. 1. Symptoms observed on affected tomato plants (cv. Castle rock) from field in Fayoum and Beni Suef governorates: A – wilt and sudden death of tomato plants; B – root rot, crown rot and stem rot; C – stem rot and fruit drop

of wilting, dead plant, root rot with crown and stem rot (Fig. 1A–C). In the surveyed fields, symptoms of root rot and stem rot could be observed as wilting symptoms on above ground plant parts (Fig. 1A). The outbreak of these symptoms occurred in a hot, wet summer, during August 2016, in Fayoum and Beni Suef governorates in northern upper Egypt. The results revealed that diseased plants having the above symptoms were prevalent and severe losses in established plants were found (approximately 35% of the affected plants) in both governorates (Table 1). A total of 16 fungal isolates was isolated from root and stem tissues of tomato plants displaying the above symptoms (Table 2). These isolates were characterized, classified and tentatively identified as belonging to six species, namely: *A. solani*, *Ch. globosum*, *F. solani*, *F. oxysporum*, *Pythium* sp. and *R. solani*. Isolates of *Pythium* sp., which were detected for the first time on tomato plants were more prevalent than the other five species and a high frequency of occurrence was recorded (Table 2).

Pathogenicity test

To prove the pathogenic ability of the recovered fungal isolates, different infestation methods were performed. Observation of the symptoms showed significant

Table 1. Percentages of diseased tomato plants showing symptoms of wilt and sudden death disease, in Bani-Suef and Fayoum governorates, Egypt in August, 2016

Governorate	Area of farm [ha]	Disease incidence [%]	Stage of growth	Symptoms
Bani-Suef	4	28.0	fruiting	root rot and stem blight
Bani-Suef	2	22.0	fruiting	root rot and stem blight
Bani-Suef	3	35.0	vegetative	root rot
Fayoum	2.5	28.0	fruiting	root rot and stem blight
Fayoum	4	20.0	fruiting	root rot and stem blight
Fayoum	2	30.0	vegetative	root rot

Table 2. Frequency of fungal isolates associated with tomato samples showing symptoms of wilt and sudden death disease

Isolated fungi	Frequency of occurrence [%][a]	
	stem parts	root parts
Pythium sp. (P1)	20.0 ± 2.04 b	18.3 ± 0.47 b
Pythium sp. (P2)	20.0 ± 1.63 b	27.7 ± 0.85 a
Pythium sp. (P3)	25.0 ±1.91 a	28.0 ± 0.40 a
Fusarium solani (Fs1)	2.0 ± 0.40 de	3.0 ± 0.00 d
F. solani (Fs2)	2.0 ± 0.70 de	3.0 ± 0.40 d
F. solani (Fs3)	2.0 ± 0.00 de	3.0 ± 0.40 d
F. solani (Fs4)	2.0 ± 0.70 de	0.0 ± 0.00 e
F. oxysporum (Fo1)	0.0 ± 0.00 e	5.0 ± 0.40 c
F. oxysporum (Fo2)	0.0 ± 0.00 e	5.0 ± 1.68 c
F. oxysporum (Fo3)	3.0 ± 0.70 de	0.0 ± 0.00 e
Rhizoctonia solani (Rs1)	7.0 ± 1.77 c	2.0 ± 0.00 d
R. solani (Rs2)	4.0 ± 0.40 d	3.0 ± 0.70 d
R. solani (Rs3)	4.0 ± 0.00 d	2.0 ± 0.40 d
Alternaria solani (As1)	3.0 ± 0.40 de	0.0 ± 0.00 e
Chaetomium globosum (Cg1)	3.0 ± 0.40 de	0.0 ± 0.00 e
Ch. globosum (Cg2)	3.0 ± 0.00 de	0.0 ± 0.00 e

[a] approximately 300 damaged tomato plants displaying symptoms of root rot, crown rot and stem rot were collected from commercial open fields in Fayoum and Beni Suef governorates, Egypt in August, 2016. The root system and foliage system of diseased tomato plants were separated, using a sterilized scalpel, and both organs were used for isolation trials

Different letters are considered significantly different by Duncan test at $p < 0.05$. Arcsine square root-transformed data for frequency of occurrence (%) were determined for statistical analysis; however, untransformed data are presented. In each column, means of ±SE is shown

differences between the isolated fungi for their pathogenicity to tomato plants. Isolates of *A. solani* and *Ch. globosum* were non-pathogenic to tomato plants (Tables 3, 4). Isolates of *F. solani*, *F. oxysporum*, and *R. solani* incited damping-off and root rot diseases while *Pythium* sp. isolates incited damping-off, root rot, sudden death, stem rot and fruit rot diseases (Tables 3, 4; Fig. 2). *Pythium* sp. isolates were found to cause higher percentages of damping-off and dead plants due to root rot diseases than the other isolates (Tables 3, 4). Root rot symptoms caused by *Pythium* sp. isolates occurred within a few days following planting of tomato seedlings (Fig. 2B–D). It was observed that the percentages of dead plants due to root rot infection decreased with the increasing of plant age. Meanwhile, the growth criteria, i.e. plant biomass (mg) of the raised seedlings (Tables 3, 4) were drastically affected by *Pythium* sp. isolates and to a lesser extent by *F. solani*, *F. oxysporum*, and *R. solani* isolates. However, inoculation of soil with *Ch. globosum* isolates resulted in a significant increase in plant biomass. The incidence of permanent wilt of tomato plants (sudden death disease) 4 and 7 days after flood water was removed is listed in Table 5. In soil infested with *Pythium* sp. isolates, over 40.0% of the tomato plants showed wilt symptoms 4 days after flood water was removed. Seven days after drainage, over 70.0% of the plants showed permanent wilt symptoms (Fig. 2E). In the non-infested control and those infested by other fungal isolates, 2.5 to 7.5% of the plants showed wilt symptoms, which was significantly lower than the inoculated treatments with *Pythium* sp. isolates (Table 5). The pathogenicity test performed by inoculating the stems of tomato seedlings revealed that water-soaked lesions and seedling blight were visible on the stems of inoculated plants with *Pythium* sp. isolates only 3 days after inoculation (Fig. 2F). Six days after stem inoculation, lesions turned dark brown and the affected plants wilted or died. Control plants and those inoculated by other fungal isolates remained asymptomatic. Finally, symptoms of water-soaked lesions with cottony mycelium cousin were visible on tomato fruits inoculated only with *P. aphanidermatum* isolates (Fig. 2G). The fungus-like organism *Pythium* sp. was successfully isolated from symptomatic plants, fulfilling Koch's postulates.

Identification of the causal pathogen

A total of three *Pythium* isolates recovered from diseased tomato plants were morphologically similar to

Table 3. Emergence of tomato seedlings and percentages of damping-off disease in soil artificially infested with different fungal isolates recovered from tomato samples

Isolated fungi	Emergence, damping-off and seedling biomass[a]		
	emergence [%]	damping-off [%]	seedling biomass [mg]
Pythium sp. (P1)	60.0 ± 0.00 f	62.5 ± 4.18 b	54.8 ± 0.28 j
Pythium sp. (P2)	40.0 ± 0.00 h	81.3 ± 6.25 a	55.1 ± 0.42 j
Pythium sp. (P3)	50.0 ± 0.00 g	80.0 ± 0.00 a	50.4 ± 0.14 k
Fusarium solani (Fs1)	70.0 ± 0.00 e	49.9 ± 4.12 c	61.8 ± 0.51 i
F. solani (Fs2)	70.0 ± 0.00 e	42.8 ± 0.00 cd	62.3 ± 1.37 i
F. solani (Fs3)	70.0 ± 0.00 e	28.6 ± 0.00 e	64.1 ± 0.35 h
F. solani (Fs4)	70.0 ± 0.00 e	46.3 ± 3.57 c	64.9 ± 0.70 gh
F. oxysporum (Fo1)	80.0 ± 0.00 b	31.3 ± 3.60 e	71.3 ± 0.62 d
F. oxysporum (Fo2)	77.5 ± 2.50 bc	25.9 ± 0.90 e	68.7 ± 0.92 e
F. oxysporum (Fo3)	75.0 ± 2.88 cd	26.8 ± 1.03 e	71.0 ± 0.70 d
Rhizoctonia solani (Rs1)	70.0 ± 0.00 e	35.7 ± 7.12 de	67.8 ± 0.41 ef
R. solani (Rs2)	72.5 ± 2.50 de	31.3 ± 3.94 e	67.4 ± 0.66 ef
R. solani (Rs3)	75.0 ± 2.88 cd	33.1 ± 5.71 de	66.4 ± 0.16 fg
Alternaria solani (As1)	96.3 ± 0.47 a	00.0 ± 0.00 f	77.8 ± 0.78 c
Chaetomium globosum (Cg1)	96.3 ± 0.62 a	00.0 ± 0.00 f	85.3 ± 0.27 a
Ch. globosum (Cg2)	95.5 ± 0.86 a	00.0 ± 0.00 f	84.5 ± 0.19 a
Control	96.3 ± 0.62 a	00.0 ± 0.00 f	80.3 ± 0.18 b

[a] the experimental period was 30 days after sowing. During the experimental period, germination percentages were recorded 7 days after sowing. The incidence of post-emergence damping-off disease was recorded during the experimental period based on the symptoms noticed on diseased plants up to 30 days after sowing. Average weight of alive seedlings in each treatment was also recorded 30 days after sowing

Different letters are considered significantly different by Duncan test at $p < 0.05$. Arcsine square root-transformed data for emergence (%) and damping-off (%) were determined for statistical analysis; however, untransformed data are presented. In each column, means of ±SE is shown

Table 4. Dead plants due to root rot disease produced by different fungal isolates in tomato plants and average plant biomass [g] on the last day of the greenhouse experiment (45 days after transplanting)

Isolated fungi	Dead plants [%] due to root rot and plant biomass[a]	
	root rot	plant biomass [g]
Pythium sp. (P1)	85.0 ± 2.89 b	12.63 ± 0.34 e
Pythium sp. (P2)	95.0 ± 2.89 a	12.33 ± 0.28 e
Pythium sp. (P3)	82.5 ± 2.50 b	12.43 ± 0.15 e
Fusarium solani (Fs1)	32.5 ± 2.50 d	15.33 ± 0.11 c
F. solani (Fs2)	30.0 ± 0.00 d	15.48 ± 0.16 c
F. solani (Fs3)	32.5 ± 2.50 d	15.25 ± 0.15 c
F. solani (Fs4)	30.0 ± 0.00 d	15.40 ± 0.23 c
F. oxysporum (Fo1)	15.0 ± 2.89 e	15.60 ± 0.22 c
F. oxysporum (Fo2)	17.5 ± 2.50 e	15.53 ± 0.19 c
F. oxysporum (Fo3)	15.0 ± 2.89 e	15.40 ± 0.23 c
Rhizoctonia solani (Rs1)	42.5 ± 2.50 c	14.15 ± 0.22 d
R. solani (Rs2)	45.0 ± 2.89 c	14.43 ± 0.17 d
R. solani (Rs3)	42.5 ± 2.50 c	14.45 ± 0.16 d
Alternaria solani (As1)	0.00 ± 0.00 f	16.55 ± 0.19 b
Chaetomium globosum (Cg1)	0.00 ± 0.00 f	17.05 ± 0.23 ab
Ch. globosum (Cg2)	0.00 ± 0.00 f	17.40 ± 0.26 a
Control	0.00 ± 0.00 f	16.58 ± 0.06 b

[a] the experimental period was 30 days after transplanting. During the experimental period, disease incidence as the percentages of dead plants due to root rot was recorded. Average plant biomass of living plants in each treatment was also recorded at the end of experiment

Different letters are considered significantly different by Duncan test at $p < 0.05$. Arcsine square root-transformed data for disease incidence (%) were determined for statistical analysis; however, untransformed data are presented. In each column, means of ±SE error is shown

each other. Based on their morphology, all isolates were identified as *P. aphanidermatum* (Fig. 3A–C). The identification characters considered correspond to those in previous studies (van der Plaats-Niterink *et al*. 1981). Total DNA of the three *Pythium* isolates was extracted and checked using agarose gel electrophoresis (Figs. 4, 5). The 18S rRNA gene was amplified using ITS 4 and DC 6 as forward and reverse primers in a thermal cycler. By comparing the PCR products with the DNA marker, the size of PCR product was 1310 Pb. The purified PCR products were sequenced. The obtained sequencing data were compared with the global recorded database in the National Centre for Biotechnology Information (NCBI) using BLAST program. The similarity degree of this isolate with *P. aphanidermatum* was the highest (95%). The sequences of these strains were uploaded to GenBank and recorded under accession numbers MF356676, MF356677 and MF356678. The phylogenetic tree showed that these strains were very close to the type strains of *P. aphanidermatum* genera deposited in the Culture Collection Centre of NCBI (Fig. 6).

Biogenic synthesis of silver nanoparticles (AgNPs)

Silver nanoparticles were produced using the *F. oxysporum* strain. The production of nanosized particles was detected by spectrophotometer and the curve of scanning is illustrated in Figure 7. The results showed that one peak was recorded at 385 nm. The size and formation of nanoparticles was characterized by TEM and the picture produced is illustrated in Figure 8. The size of the spherical particles ranged from 10 to 30 nm.

In vitro inhibitory effect of silver nanoparticles (AgNPs) against *Pythium aphanidermatum* (Pa2)

Inhibitory effect tests of AgNPs were performed against the most virulent isolate of *P. aphanidermatum* (Pa2) on PDA plates treated with different concentrations of AgNPs (40, 80, 120, 160 and 200 mg · l^{-1}). For the concentration of 40 mg · l^{-1}, AgNPs showed a mild growth-inhibitory effect and there was no statistically significant inhibitory effect compared with the control (Table 6; Fig. 8). Comparing with the control, other AgNPs concentrations showed growth inhibition against *P. aphanidermatum* (Pa2), and significant growth inhibition was observed with 80 mg · l^{-1} (Table 6; Fig. 9A–F). The lowest inhibition level (26.1%) was noted on PDA treated with 80 mg · $l^{-1}$of AgNPs. The highest level of inhibition (87.8%) was observed with 200 mg · l^{-1} of AgNPs. In addition, the zoospores treated by AgNPs showed significant germination inhibition after encystment compared to control after

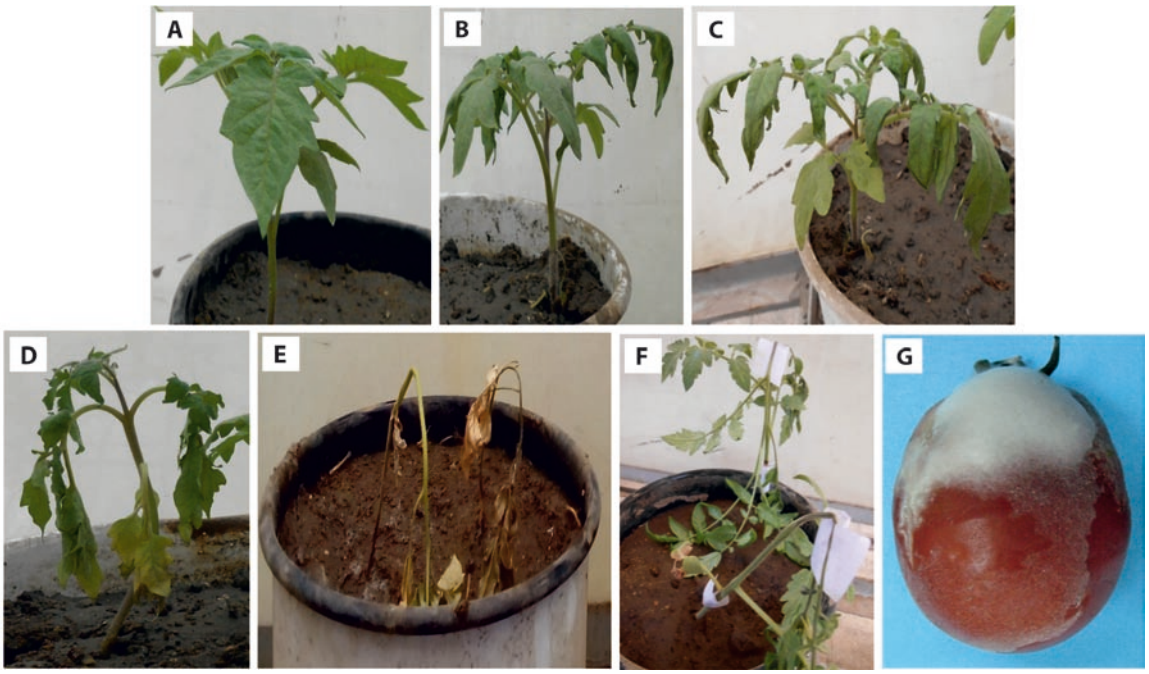

Fig. 2. Symptoms observed on tomato parts upon various methods of inoculation with *Pythium aphanidermatum*. Soil infestation was conducted two weeks before tomato transplanting: A – healthy plant, B – beginning of root rot, C – root rotted plants, D – sever root rot. Soil infestation was conducted two weeks after tomato transplanting: E – wilt and sudden death symptom. Stem inoculation method: F – water soaked lesions advanced on the stems inoculated with plugs from agar cultures of *P. aphanidermatum*. Fruit inoculation method: G – cottony mycelium developed on inoculated tomato fruit with plugs from agar cultures of *P. aphanidermatum*

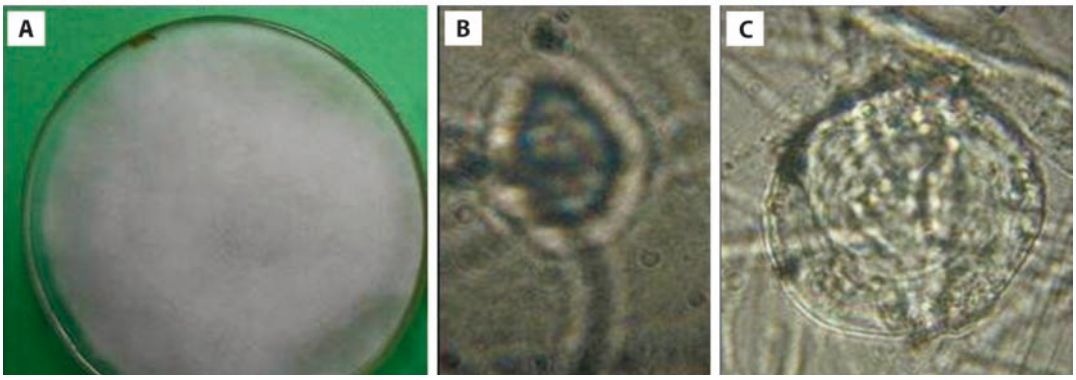

Fig. 3. Morphology of *Pythium aphanidermatum*: A – surface side of *P. aphanidermatum* isolate after 5 days of growth on PDA; B – sporangium and sporangiophors; C – oospore

24 h (Table 7). All AgNPs concentrations showed statistically significant inhibitory effects compared with the control (Table 7). The lowest level of inhibition (25.0%) was observed with a 40 mg · l^{-1} concentration of AgNPs. The highest level of inhibition (78.0%) was observed with a 200 mg · l^{-1} concentration of AgNPs (Table 7).

Greenhouse and field experiments

The results illustrated in Table 8 demonstrate that all AgNPs treatments suppressed the incidence of tomato death due to root rot compared to untreated control under greenhouse and field conditions. The results demonstrate that the method of root dipping plus soil drenching with AgNPs (200 mg · l^{-1}) was the best treatment against root rot followed by individual treatment of root dipping and soil drenching. On the other hand, all the growth parameters assessed in the greenhouse (Table 9) and in the field (Table 10) were increased in treated tomato plants in comparison to the control. The data demonstrated that the highest growth was recorded in tomato plants treated with AgNPs (200 mg · l^{-1}) by the method of root dipping plus soil drenching.

Table 5. Effect of different fungal isolates on incidence of permanent wilt (sudden death disease) of tomato plants 4 and 7 days after flood water was removed in a greenhouse experiment conducted in August, 2017

Isolated fungi	Permanent wilt (sudden death disease) of tomato [%][a]	
	4 days after flooding	7 days after flooding
Pythium sp. (P1)	40.0 ± 0.00 a	82.5 ± 2.50 a
Pythium sp. (P2)	42.5 ± 2.50 a	82.5 ± 2.50 a
Pythium sp. (P3)	42.5 ± 2.50 a	80.0 ± 0.00 a
Fusarium solani (Fs1)	05.0 ± 2.89 b	07.5 ± 2.50 bc
F. solani (Fs2)	05.0 ± 2.89 b	07.5 ± 2.50 bc
F. solani (Fs3)	05.0 ± 2.89 b	07.5 ± 2.50 bc
F. solani (Fs4)	05.0 ± 2.89 b	07.5 ± 2.50 bc
F. oxysporum (Fo1)	05.0 ± 2.89 b	10.0 ± 0.00 b
F. oxysporum (Fo2)	05.0 ± 2.89 b	10.0 ± 0.00 b
F. oxysporum (Fo3)	05.0 ± 2.89 b	10.0 ± 0.00 b
Rhizoctonia solani (Rs1)	05.0 ± 2.89 b	05.0 ± 2.89 bc
R. solani (Rs2)	05.0 ± 2.89 b	05.0 ± 2.89 bc
R. solani (Rs3)	05.0 ± 2.89 b	05.0 ± 2.89 bc
Alternaria solani (As1)	05.0 ± 2.89 b	05.0 ± 2.89 bc
Chaetomium globosum (Cg1)	02.5 ± 2.50 b	02.5 ± 2.50 c
Ch. globosum (Cg2)	02.5 ± 2.50 b	02.5 ± 2.50 c
Control	05.0 ± 2.89 b	05.0 ± 2.89 bc

[a]healthy tomato seedlings (30-days-old, cv. Castle rock) were transplanted into plastic pots at the rate of 2 seedlings/pot. Six weeks later, inocula of each of the tested fungal isolates were added to the top of pots at a ratio of 10 g · pot^{-1} and the pots were flooded immediately for 48 hours at 30°C (±2°C) after inoculation (Le *et al*. 2003). The number of wilted plants from all treatments was recorded 4 and 7 days after flooding water was removed

Different letters are considered significantly different by Duncan test at $p < 0.05$. Arcsine square root-transformed data for disease incidence [%] were determined for statistical analysis; however, untransformed data are presented. In each column, means of ±SE is shown

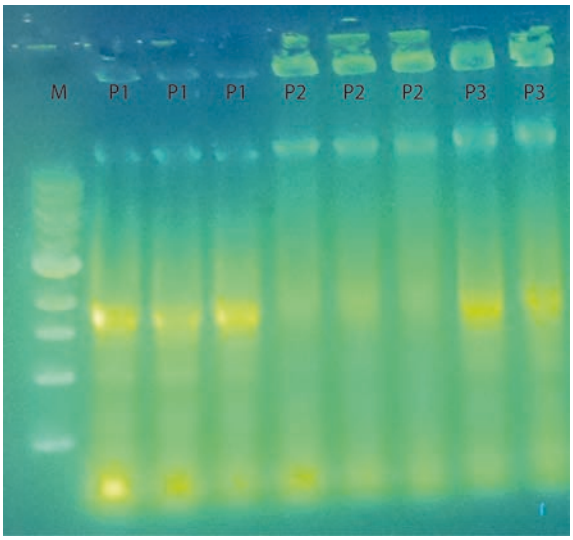

Fig. 4. Agarose gel electrophoresis for total genomic DNA of *Pythium aphanidermatum* isolates: M – DNA ladder (1kb); P1 – *Pythium* isolate 1; P2 – *Pythium* isolate 2; P3 – *Pythium* isolate 3

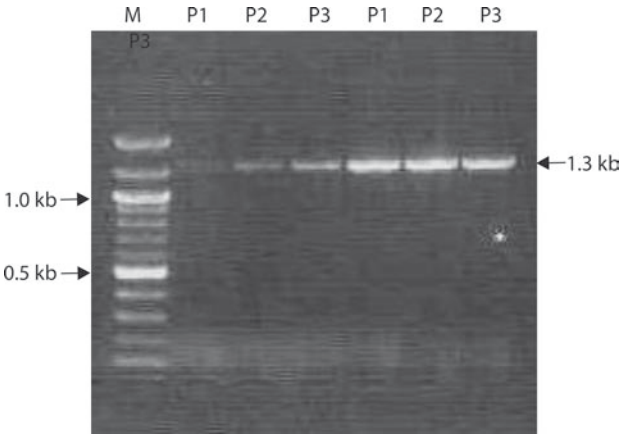

Fig. 5. Agarose gel electrophoresis for PCR products of 18S rRNA analysis of *Pythium aphanidermatum* isolates: M – DNA ladder (100 bp); P1 – *Pythium* isolate 1; P2 – *Pythium* isolate 2; P3 – *Pythium* isolate 3

Discussion

Pythium species are a worldwide threat for vegetable production causing damping-off as well as poor growth and low yields of surviving plants. Among these, *P. aphanidermatum* is one of the most serious pathogens, capable of causing catastrophic yield losses (Favrin *et al*. 1988). This fungus-like organism is known to have a high temperature optimum of 35–40°C and it is also known that temperature greatly influences infection and the subsequent damage it

Table 6. Linear growth of *Pythium aphanidermatum* (P2) in the presence of different concentrations of silver nanoparticles (AgNPs) and efficiency of treatments *in vitro*

AgNPs [mg · l^{-1}]	Linear growth of *P. aphanidermatum* and reduction	
	linear growth [mm]	reduction [%]
40	90.00 ± 0.00 a	00.0
80	66.50 ± 3.50 b	26.1
120	43.50 ± 1.06 c	51.7
160	27.00 ± 0.81 d	70.0
200	11.00 ± 0.66 e	87.8
Control	90.00 ± 0.00 a	00.0

Different letters are considered significantly different by Duncan test at $p < 0.05$. In the linear growth column, means of ± SE is shown

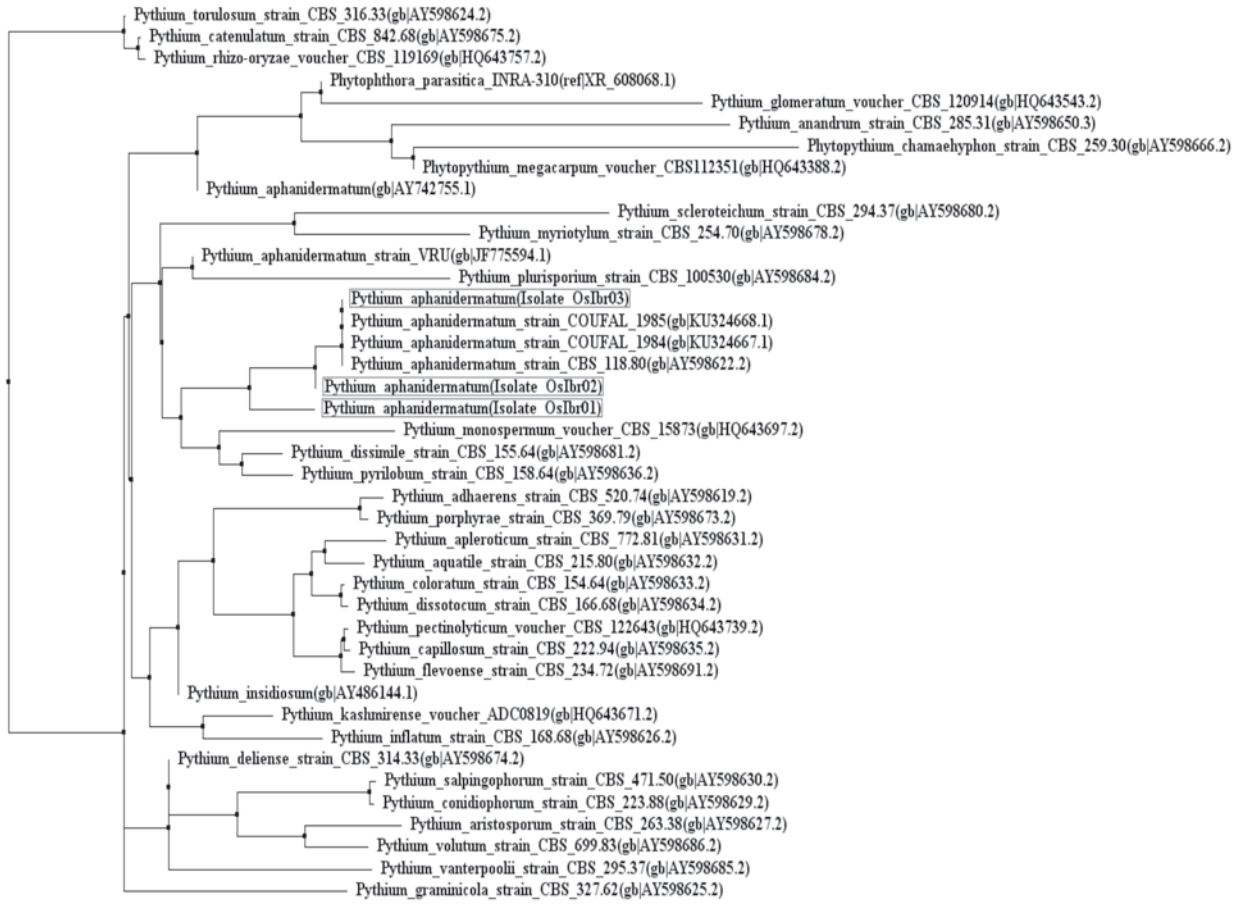

Fig. 6. Phylogenetic tree constructed from the 18SrRNA sequence of *Pythium aphanidermatum* isolates and their related strains in Gene Bank

causes to its plant host (Thomson *et al.* 1971). In the present study, isolates of *P. aphanidermatum*, recovered from tomato plants displaying symptoms of wilting, dead plant, root rot with crown and stem rot were prevalent and more pathogenic than the other associated fungal isolates. It causes damping-off, root rot, sudden death, stem rot and fruit rot. Pathogenicity tests of *P. aphanidermatum* isolates showed that these isolates caused damping-off and seedling death of up to 80.0 and 95.0% in infested soil. These results are in accordance with many researchers. Agrios (2005) reported that damping-off is the worst disease in tomato young seedlings and can kill them as well as germinating seeds. The mainly tropical distribution and pathogenicity to a wide host range is caused by *P. aphanidermatum*. It infects mainly seedling roots or root tips of older plants and is known to be a good root colonizer that consistently inhibits root growth. After that, the *P. aphanidermatum* can move through the seedling roots into the hypocotyl causing stem rot and eventually post-emergence damping-off. Manjula (2008) reported that *P. aphanidermatum* caused more than 75–90% seedling mortality in cauliflower and broccoli. Kipngeno *et al.* (2015) reported seedling damping-off

Table 7. Inhibition of zoospore germination after 24 h of incubation of a mixture of equal volumes of *Pythium aphani dermatum* (P2) and different concentrations of silver nanoparticles (AgNPs)

AgNPs [mg · l^{-1}]	Inhibition of zoospore germination [%]
40	25.00 ± 1.29 e
80	42.00 ± 0.81 d
120	50.20 ± 1.16 c
160	61.20 ± 0.66 b
200	78.00 ± 0.81 a
Control	21.00 ± 0.66 f

Different letters are considered significantly different by Duncan test at $p < 0.05$. Arcsine square root-transformed data for inhibition of zoospore germination (%) were determined for statistical analysis; however, untransformed data are presented. Means of ± SE is shown

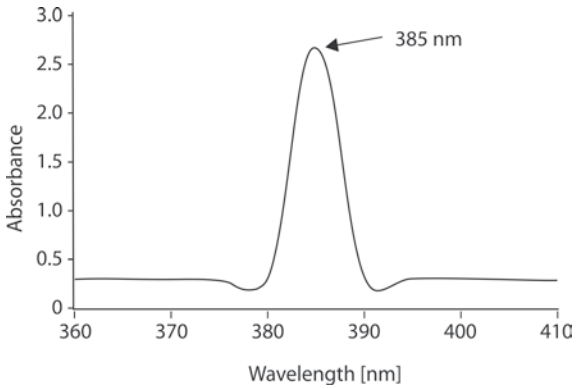

Fig. 7. Spectrophotometric scanning of biogenic silver nanoparticles

Table 8. Dead plants due to root rot disease produced by *Pythium aphanidermatum* in tomato plants in response to silver nanoparticles (AgNPs) at the concentration of 200 mg · l^{-1} on the last day of the greenhouse and field experiments

Treatment	Dead plants [%] due to root rot	
	greenhouse experiment [a]	field experiment [b]
Root dipping in AgNPs	50.00 ± 0.00 b	17.73 ± 1.03 b
Soil drenching with AgNPs	55.00 ± 2.88 b	19.78 ± 1.03 b
Root dipping plus soil drenching	37.50 ± 2.50 c	11.45 ± 1.05 c
Control	92.50 ± 1.44 a	26.05 ± 2.01 a

[a]the experimental period was 30 days after transplanting. During the experimental period, disease incidence as the percentages of dead plants due to root rot was recorded

[b]the experimental period was 60 days after transplanting. During the experimental period, disease incidence as the percentages of dead plants due to root rot, crown rot and stem rot was recorded

Different letters are considered significantly different by Duncan test at $p < 0.05$. Arcsine square root-transformed data for disease incidence (%) were determined for statistical analysis; however, untransformed data are presented. In each column, means of ±SE is shown

Table 9. Effects of silver nanoparticles (AgNPs) on plant biomass and number of branches of tomato plants grown under greenhouse conditions[a]

AgNPs treatment	Plant height [cm]	Number of leaves	Plant biomass [g]
Roots dipping (Rd)	41.75 ± 0.63 b	8.75 ± 0.25 a	18.38 ± 0.10 a
Soil drenching (Sd)	42.25 ± 0.85 b	8.75 ± 0.25 a	18.68 ± 0.16 a
Rd + Sd	45.00 ± 0.82 a	9.25 ± 0.25 a	18.88 ± 0.39 a
Control	32.25 ± 0.75 c	7.25 ± 0.25 b	12.43 ± 0.10 b

[a]average plant height (cm), number of leaves and plant biomass of living seedlings in each treatment were determined 30 days after transplanting
Different letters are considered significantly different by Duncan test at $p < 0.05$. In each column, means of ±SE is shown

Table 10. Effects of silver nanoparticles (AgNPs) on average plant height, average number of branches of tomato plants grown under field conditions[a]

AgNPs treatment	Plant height [cm]	No. of branches
Roots dipping (Rd)	108.75 ± 0.48 b	9.0 ± 0.00 b
Soil drenching (Sd)	109.75 ± 0.25 ab	9.0 ± 0.00 b
Rd + Sd	110.50 ± 0.29 a	9.8 ± 0.25 a
Control	099.50 ± 0.29 c	8.0 ± 0.00 c

[a]average plant height (cm) and number of branches of alive plants in each treatment were recorded 60 days after transplanting
Different letters are considered significantly different by Duncan test at $p < 0.05$. In each column, means of ± SE is shown

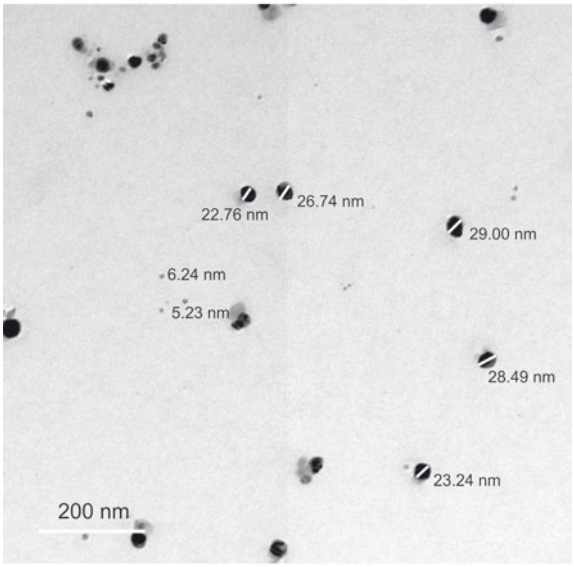

Fig. 8. Transmission electron microscopy (TEM) image of spherical biogenic silver nanoparticles (AgNPs) size and shape

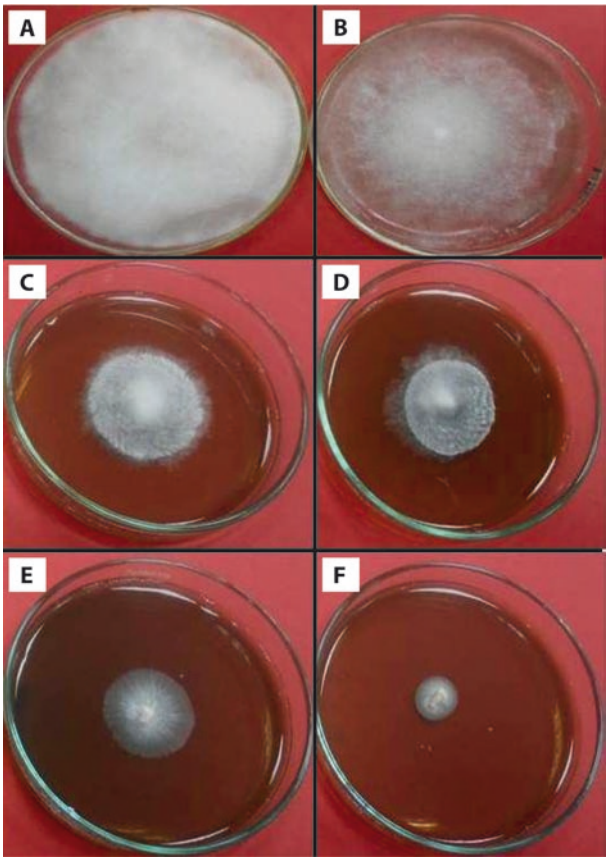

Fig. 9. Inhibition effects of silver nanoparticles (AgNPs) against *Pythium aphanidermatum* (Pa2) on PDA, *in vitro*: A – control, B – 40 mg · l^{-1} AgNPs, C – 80 mg · l^{-1} AgNPs, D – 120 mg · l^{-1} AgNPs, E – 160 mg · l^{-1} AgNPs, F – 200 mg · l^{-1} AgNPs

disease of tomato caused by *P. aphanidermatum* in Kenya. They added that it can cause up to a 30% decrease in seedlings. Many researchers all over the world have reported that *P. aphanidermatum* is a major causal agent of damping-off, root rot and wilt of tomato (Jayaraj *et al.* 2005; Christy Jeyaseelan *et al.* 2012; Kipngeno *et al.* 2015). In Egypt, other soil-borne fungi are reported to attack tomato plants causing root rot and crown rot diseases of tomato (Ghonim 1999; Saad 2006; Morsy *et al.* 2009; Mohamed *et al.* 2015; Hamza *et al.* 2016; Shenashen *et al.* 2017). In the present study we found that *P. aphanidermatum* is an additional pathogen to tomato grown in Egypt causing damping-off, root rot, crown rot, stem decay and fruit rot diseases. The present study proved that *P. aphanidermatum* causes severe damage to tomato plants grown under greenhouse conditions following flooding. Such results are in harmony with those obtained by Le *et al.* (2003), who reported that *P. aphanidermatum* plays a key role in sudden death disease of tomato.

Silver nanoparticles have been evaluated recently against plant pathogens *in vitro* only (Min *et al.* 2009; Kim *et al.* 2012). They concluded that AgNPs have *in vitro* antifungal activity against different plant pathogens at several levels. Findings from the current investigation demonstrated that AgNPs were very effective against *P. aphanidermatum* (Pa2). The results showed that AgNPs are capable of inhibiting both the linear growth of mycelium and germination of zoospores of the pathogen *in vitro*. The inhibition activity was increased with greater AgNPs concentrations. This may have been due to the high density of nanosized silver in solution which was able to saturate and cohere the fungal hyphae degrading of DNA and proteins (Park *et al.* 2006). It also inactivated the expression of ribosomal subunit proteins as well as certain other cellular proteins and ATP production (Yamanaka *et al.* 2005). Ag^{+} primarily affects the function of enzymes in the respiratory chain (Bragg and Rainnie 1974). It may directly attach to and penetrate through the cell membrane to kill mycelium-like organs and spores. The primary antifungal property is attributable to silver ions (Jo *et al.* 2009). The nanoparticles of Ag are likely to be involved in affecting spores (Jo *et al.* 2009). The nanosized silver showed significant activity against *P. aphanidermatum* in both greenhouse and field experiments. The results demonstrate that the method of root dipping plus soil drenching with AgNPs (200 mg · l^{-1}) was the best treatment. It was statistically followed by individual treatment of root dipping and soil drenching. The antifungal mechanism of AgNPs may be due to the fact that the formation of free radicals produced from the nanoparticles could disturb the lipid membranes and then finally disrupt the membrane functions (Kim *et al.* 2007). Sondi and Salopek-Sondi (2004) have presented a new finding that the membrane could be deteriorated by the formation of pits on the surface of the cell wall membrane of microorganisms. The formation of pits on the membrane leads to increased permeability and irregular transport that results in the death of the cells.

Conclusions

The present study concluded that *P. aphanidermatum* is an additional pathogen to tomato grown in Egypt causing damping-off, root rot, wilt with sudden death, crown rot, stem rot and fruit rot diseases. The results also clearly demonstrated that biologically synthesized AgNPs are hopeful antifungal agents against *P. aphanidermatum in vitro* and *in vivo*. So, the green-synthesized silver nanoparticle is a good facility, which is easily produced and extensively useful in agricultural applications. More work is required to explain any possible toxicity due to AgNPs application on edible vegetables.

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