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Biological Control of *Pythium* Damping-off of Bush Okra Using Rhizosphere Strains of *Pseudomonas fluorescens*

Hani M. A. Abdelzaher[1]*, M. M. Imam[1], M. A. Shoulkamy[1] and Y. M. A. Gherbawy[2]

[1]*Department of Botany, Faculty of Science, El-Minia University, El-Minia 61519, Egypt*
[2]*Department of Botany, Faculty of Science, South Valley University, Qena, Egypt*

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A severe damping-off disease of bush okra caused by *Pythium aphanidermatum*, was diagnosed in plastic houses in Der Attia village, 15 km southwest of El-Minia city, Egypt, during the winter of 2001. Bush okra seedlings showed low emergence with bare patches inside the plastic houses. Seedlings that escaped pre-emergence damping-off showed poor growth, stunting and eventually collapsed. Examination of the infected tissues confirmed only *Pythium aphanidermatum*, showing its typical intercalary antheridia, and lobulate zoosporangia. *P. aphanidermatum* was shown to be pathogenic on bush okra under pot and field experiments. Bacteria making inhibition zones against the damping-off fungus *P. aphanidermatum* were selected. Agar discs from rhizosphere soil of bush okra containing colonies were transferred onto agar plate culture of *P. aphanidermatum*. After 2 days of incubation, colonies producing clear zones of non-*Pythium* growth were readily detected. The two bacteria with the largest inhibition zones were identified as *Pseudomonas fluorescens*. Bush okra emergence (%) in both pot and plastic houses experiments indicated that disease control could be obtained by applying *P. fluorescens* to the soil or coating the bacteria to the bush okra seeds before sowing. In the plastic houses, application of the bacteria onto *Pythium*-infested soil and sowing bush okra seeds dressed with bacteria gave 100% emergence. In addition, This was the first reported disease of bush okra by this oomycete in Egypt.

KEYWORDS: Biological control, Bush okra, Damping-off, *Pseudomonas fluorescens*, *Pythium aphanidermatum*

Bush okra, jew's mallow or jute mallow (*Corchorus olitorius* L.) is considered to be one of the popular tropical leaf vegetables in Africa, Asia, and some parts of the Middle East. It is a very popular leaf vegetable in Egypt (Oomen and Grubben, 1978). The famous Egyptian dish (molokiia) is a favorite for most Egyptians. Bush okra is primarily known as a fiber crop, however, some types have been cultivating as a mucilaginous tropical leaf vegetable. In Egypt, bush okra is cultivated from March to November. In the winter, farmers plant this crop in plastic houses in many areas throughout the country.

A severe pre-emergence damping-off disease of bush okra was recognized in many plastic houses in Der Attia village 15 km south west of El-Minia city, Egypt in November 2001. The disease was characterised by low emergence of bare patches inside the plastic houses. Plants that escaped from the pre-emergence damping-off showed poor growth, stunting and eventually collapsed above the soil surface.

Pythium aphanidermatum was not previously recorded as a pathogen to bush okra despite the wide host range of this fungus (Elnaghy *et al.*, 2002). It is a cosmopolitan pathogen with a wide host range especially in the warm regions (Elnaghy *et al.*, 2002) but also occurs in cool climates where crops are grown inside green houses. It is an aggressive species of *Pythium*, causing damping-off, root and stem rots, wilts, blights and fruit rot (Plaats-Niterink, 1981).

Biological control is becoming a promising component of plant disease management. Chemical control methods can represent risks to the environment as well as encourage fungicide-resistant mutants of the pathogens. For these reasons, fungicides are being registered owing to concerns about their safety and environmental impacts. Furthermore, chemicals that are registered for use may be unavailable to growers because of pressures and concerns from the general public (Harman, 1991). Therefore, other ways to control pathogenic species of *Pythium* are needed. Currently, increasing attention has been paid to biological control through the use of antagonistic bacteria (Abdelzaher, 2003; Abdelzaher and Elnaghy, 1998; Paulitz and Bélanger, 2001; Ryder *et al.*, 1999). Some fluorescent pseudomonads are referred to as plant growth-promoting rhizobacteria and their effectiveness in controlling a number of plant diseases caused by soil-borne pathogens has been widely documented (Abdelzaher and Elnaghy, 1998; Glick, 1995; Jacques *et al.*, 1993; O'Sullivan and O'Gara, 1992).

This paper describes the first occurrence of damping-off of bush okra caused by *P. aphanidermatum* in Egypt and assesses the possibility of using antagonistic rhizospheric strains of *Pseudomonas fluorescens* (Trevisan) to control this disease.

*Corresponding author <E-mail: abdelzaher@link.net>

Materials and Methods

Isolation of *Pythium* spp. from damped-off bush okra seedlings. Damped-off bush okra germinating seeds and seedlings were collected from plastic houses at Der Attia village, El-Minia, Egypt, in November 2001. The damped-off bush okra seedlings (basal part of the stem and the roots) were rinsed in ethanol (50%, v/v) for 30 s, transferred to tap water, followed by sterile tap water, blotted with sterile filter paper, and then transferred to VP3 (Ali-Shtayeh *et al.*, 1986) selective medium: sucrose, 20 g/litre; corn meal agar, 17 g/litre; agar, 23 g/litre; $CaCl_2$, 0.01 g/litre; $MgSO_4 \cdot 7H_2O$, 0.01 g/litre; $ZnCl_2$, 0.001 g/litre; micro-elements: $CuSO_4 \cdot 5H_2O$, 0.02 mg/litre; MoO_3, 0.02 mg/litre; $MnCl_2$, 0.02 mg/litre; $FeSO_4 \cdot 7H_2O$, 0.02 mg/litre; antibiotics: pimarcin, 5 mg/litre; vancomycin, 75 mg/litre; penicillin, 50 mg/litre; pentachloronitrobenzene, 130 mg/litre and thiamine-HCl, 100 µg/litre. The tissues were incubated at 20°C for 3 days or until colonies appeared. Hyphal tips were transferred to 2.5~3% water agar (WA) and incubated at 20°C to obtain a colony ca. 1 cm diameter. The agar medium was inverted and incubated until the colony reached the edge of the Petri dish. Slivers of agar containing single hyphal tips were removed from the margin of the colony and transferred to corn meal agar (CMA) (Plaats-Niterink, 1981) slants for storage. Hyphal tips were also transferred to CMA + 500 µg/*ml* wheat germ oil to stimulate the formation of sexual structures. Pieces from 7-day-old colonies incubated at 25°C were transferred to nutrient broth (NB, Difco Manual, 1953) to confirm the absence of bacteria.

Fungal isolation from the rhizosphere of bush okra. Soil from the rhizosphere of bush okra plants was transferred to VP3 media and fungi isolated identified using the key of *Pythium* species (Plaats-Niterink, 1981; Dick, 1990) and others (Abdelzaher *et al.*, 1997a, b; Abdelzaher and Elnaghy, 1998).

Taxonomic identification of *Pythium* spp. *Pythium* was identified to the species level on the bases of sexual and asexual structures (Abdelzaher, 1999; Dick, 1990; Plaats-Niterink, 1981; Waterhouse, 1967, 1968). Sporangial formation was induced by placing young leaf blade (Dick, 1990) segments (1 cm) of maize plants colonised by *Pythium* species in Petri-dishes (7 cm diam.) containing 10 *ml* of sterilised distilled water and incubated at 5, 10, 15, 20, 25, 30, and 30°C (Abdelzaher *et al.*, 1997c). Sexual reproduction is commonly abundant in water cultures. Identification was based on studies of both water cultures and solid agar media such as CMA and potato carrot agar (PCA), each supplemented with 500 µg/*ml* wheat germ oil. All of the description criteria were only based on one isolate of *Pythium* species (Isolate 1), however, Isolate 2 was identical by using the same identification criteria. Thirty measurements were made of each structure for the isolated *Pythium*. Antheridia and sporangia form rapidly but may degenerate, therefore, cultures were observed 12 h after inoculation and periodically until all possible characters had been observed.

Temperature-growth relations. CMA was inoculated with 5 mm diam. discs from stock cultures on WA. All Petri dishes were first incubated at 25°C for 24 h then transferred to a range of temperatures from 2 to 43°C; there were five dishes (replicates) per treatment.

Isolation of antagonistic bacteria from the rhizosphere of bush okra. One *ml* aliquots of diluted rhizosphere (1/100, w/v) soil were transferred to each of 10 dishes of sodium albuminate agar (2.5% agar (Koike, 1967), with some modification (suitable dilution) and incubated at 30°C for 4 days. At the same time, *P. aphanidermatum* colonies were cultivated on WA at 30°C in the darkness. Colonies of bacteria were then inverted onto *P. aphanidermatum*. The sodium albuminate medium stimulated *P. aphanidermatum* growth sufficiently 48 h after at 30°C to make the inhibition zones around the colonies distinct when the plates were held against the light. Inhibition zones were determined and the bacteria responsible for them were transferred to sodium albuminate agar plates for purification and identification.

For identification of bacteria, various assay procedures were used. Reference *Pseudomonas* strains used were held as part of the culture collection from Microbiology Department, University of Kaiserslautern, Germany. The source of these strains and details of the DSM *Pseudomonas* reference strains (Deutsche Samlung von Mikroorganismen, Brounschweig, Germany) used for comparative purposes. The biochemical identification of the *Pseudomonas* isolates were carried out in accordance with Bergey's Manual of Systematic Bacteriology (Palleroni, 1984). Cell form, Gram reaction, motility and flagellar morphology were determined by using 0.5% glucose nutrient agar containing 0.5% glucose, 0.5% peptone, 0.5% beef extract, 0.25% NaCl and 1.5% agar at 30°C. The gram staining was carried out by the Hucker-Conn modification (1923). Motility was determined by using the hanging drop method and flagellar morphology was determined by the staining method of Kodaka *et al.* (1982). Production of fluorescent pigment was examined on King A and King B media. Growth on MacConkey and SS agar was determined 24 h after incubation. The production of catalase activity was detected by the production of bubbles in 3% hydrogen peroxidase solution. The oxidase test was performed with cytochrome oxidase test paper (Nissui Seiyaku, Tokyo, Japan). Acid production from sugar and sugar alcohols were tested. The reaction in the indole, Voges

Proskauer test and the hydrolysis of starch, gelatin and tween 80 were determined. Utilization of citrate was tested on simmon citrate agar. The growth at pH 7 in 6.5% NaCl at 4 and 37°C were tested. Nitrate reduction and urease activity were determined.

In addition, all strains were identified by means of API-50-CH test strips (Bio Mèrienx, Marcy-LEtoile, France). The sensitivity to penicillin was determined with the disc diffusion susceptibility test (Bauer *et al.*, 1966).

Isolation of antagonistic bacteria from non-rhizosphere soil of bush okra. One *ml* aliquots of diluted non-rhizosphere (1/100, w/v) soil were transferred to each of 10 dishes of sodium albuminate agar (2.5% agar (Koike, 1967) with some modifications) and incubated at 30°C for 4 days. The emerging *P. fluorescens* were identified and counted following the method described previously.

Antagonism between *P. aphanidermatum* and *P. fluorescens* strains on agar plates. Sixteen isolates of *P. fluorescens*, isolated from bush okra rhizosphere soil, were tested for in vitro antagonism against *P. aphanidermatum* on PDA plates. Each plate was divided into two equal halves, one half was inoculated with *P. aphanidermatum* and the other half was also inoculated with a disc of *P. fluorescens*. The tests were incubated at 30°C until a zone of inhibition was apparent.

Pathogenicity. The pathogenicity of *P. aphanidermatum* (Isolate 1) from damped-off bush okra seedlings, Isolate 2 from bush okra rhizosphere, and El-U520 which was isolated previously from rhizosphere of turnip (Elnaghy *et al.*, 2002) were tested for pathogenicity on bush okra. The method of Abdelzaher (2001) was used in which the fungal isolates were grown for 21 days in 500 *ml* Erlenmeyer flasks each containing 200 *ml* of sterile mixture of corn meal-sand (30 : 70 v/v) to which 50 *ml* distilled water was added in order to prepare the inocula. Clay loamy (50% sand, 40% clay, and 60% silt) soil was sterilised at 121°C for 2 h, aerated for at least 2 weeks at room temperature and then 100 g of the inoculum was incorporated into 900 g of the sterilised soil. Control soils were inoculated with sand-cornmeal mixture free from the pathogen. Surface-sterilised 50 seeds of bush okra were planted in infested soils in plastic pots (100 *ml* capacity and 7.5 cm diam.). Five replicate pots were used for each treatment and mean values were taken for 250 seedlings. Non-infested controls were included in all tests. Soils were watered to saturation every 3~4 days.

In the post-emergence damping-off experiments, 100 g inocula prepared as mentioned previously were spread around the seedlings after emergence.

The experiments were carried out in a growth cabinet (Precision, United States) at 30°C with 12 h photoperiod (91 μmol m^{-2} s^{-1}). Pre-emergence damping-off was determined as the difference in seedlings emergence between non-inoculated control and inoculated soil. Post-emergence damping-off was also determined as a percentage of the emerged plants to the number of diseased ones.

Effect of *P. fluorescens* on damping-off of bush okra in the pots. Autoclaved (free of soil-borne fungi) and natural (non-autoclaved, from adjacent healthy bush okra cultivated plastic houses free of *P. aphanidermatum*) clay-loamy (50% sand, 40% clay, and 60% silt) soil (pH = 6.7) was mixed with *Pythium* soil-cornmeal culture at a rate of 10% inoculum density (100 g inoculum prepared as described previously + 900 g autoclaved soil). The effects of 2 strains of *P. fluorescens* on pre-emergence damping-off caused by *P. aphanidermatum* was assessed by 2 methods; a) the two strains of *P. fluorescens* suspensions were mixed with the soil at 10^8 cells per g dry soil before sowing, b) the seeds were coated with suspension of 2 × 10^9 bacteria per *ml* + 2% (w/v) carboxymethylcellulose (CMC). Seed coating was achieved by soaking the seeds for 3 min in bacterial culture, the seeds were removed, spread in a sterile open petri-dish, and allowed to dry overnight in a laminar flow cabinet at 8~22°C. Batches of seeds were also prepared using bacterial-free CMC coating. All seeds were then used immediately in damping-off experiments. There were 50 seeds per pot and 5 pots (replicates) per treatment: (1) *Pythium* isolate 1 + bacteria-strain 1 (soil mixing); (2) *Pythium* isolate 1 + bacteria-strain 2 (soil mixing); (3) *Pythium* isolate 2 + bacteria-strain 1 (seed coating); (4) *Pythium* isolate 2+ bacteria-strain 2 (seed coating); (5) *Pythium* isolate El-U520 + bacteria-strain 1 (soil mixing); (6) *Pythium* isolate El-U520 + bacteria-strain 2 (soil mixing); (7) *Pythium* isolate El-U520 + bacteria-strain 1 (seed coating); (8) *Pythium* isolate El-U520 + bacteria-strain 2 (seed coating). After emergence (15 days), the effect of amendment of bacteria was measured by determining pre-emergence damping-off (%). Control treatments for comparison were: (1) bush okra + non-infested soil; (2) bush okra + non-infested soil + bacteria; and (3) bush okra seeds treated with bacteria/CMC + non-infested soil. The experiments were carried out in illuminated growth cabinet (Precision, United States) at 15~30°C with 12 h photoperiod (91 μmol m^{-2} s^{-1}) under humid conditions. The effect of *P. fluorescens* in post-emergence damping-off caused by *P. aphanidermatum* was undertaken in which the inocula of *P. aphanidermatum* were added after emergence and proceeded as described in the pre-emergence damping-off test.

Effect of *P. fluorescens* on damping-off of bush okra in plastic houses. A field experiment was carried out in November 2002 to test the effect of application of isolate

1 of *P. fluorescens* (El-U444) on damping-off of bush okra caused by *Pythium aphanidermatum*. Seven plastic houses which were used for cultivation of bush okra in the previous season (November, 2001) were chosen. Each plastic house had a dimension of 6 m long and 2 meter wide with two openings, directed towards north to south. The soil in each treated plastic house was clay-loamy (50% sand, 40% clay, and 60% silt) soil (pH = 6.7). The 7 plastic houses were managed according to standard farming practices in Egypt. Soil in each plastic house was prepared by ploughing and application of a composite fertilizer containing nitrogen (190 g), phosphorus (50 g); potassium (185 g); sodium (70 g); sulphur (44 g) and boron (4.5 g). Sowing density was approximately 3000 seeds per plastic house. Treatments were: (1) a plastic house containing soil free of *Pythium* (no damping-off was detected in the previous season and *P. aphanidermatum* could not be isolated); (2) a plastic house containing natural infested soil with *P. aphanidermatum* (showed damping-off in the previous season and *P. aphanidermatum* was reisolated); (3) a plastic house containing bush okra grown in *Pythium* free soil and the soil was mixed with *P. fluorescens* (El-U444) at approximately 10^{12} cells per cm soil; (4) a plastic house containing bush okra grown seeds coated with bacteri as described previously in natural soil free of *Pythium*; (5) a plastic house containing bush okra grown in artificial soil infested with *P. aphanidermatum*; (6) a plastic house containing bush okra grown in soil artificially infestated with *P. aphanidermatum* but the soil was mixed with *P. fluorescens* at approximately 10^{12} cells per cm soil; (7) a plastic house containing bush okra seeds coated by *P. fluorescens* and grown in soil artificially infestated with *P. aphanidermatum*; and (8) bush okra seeds coated *by P. fluorescens* and grown in soil

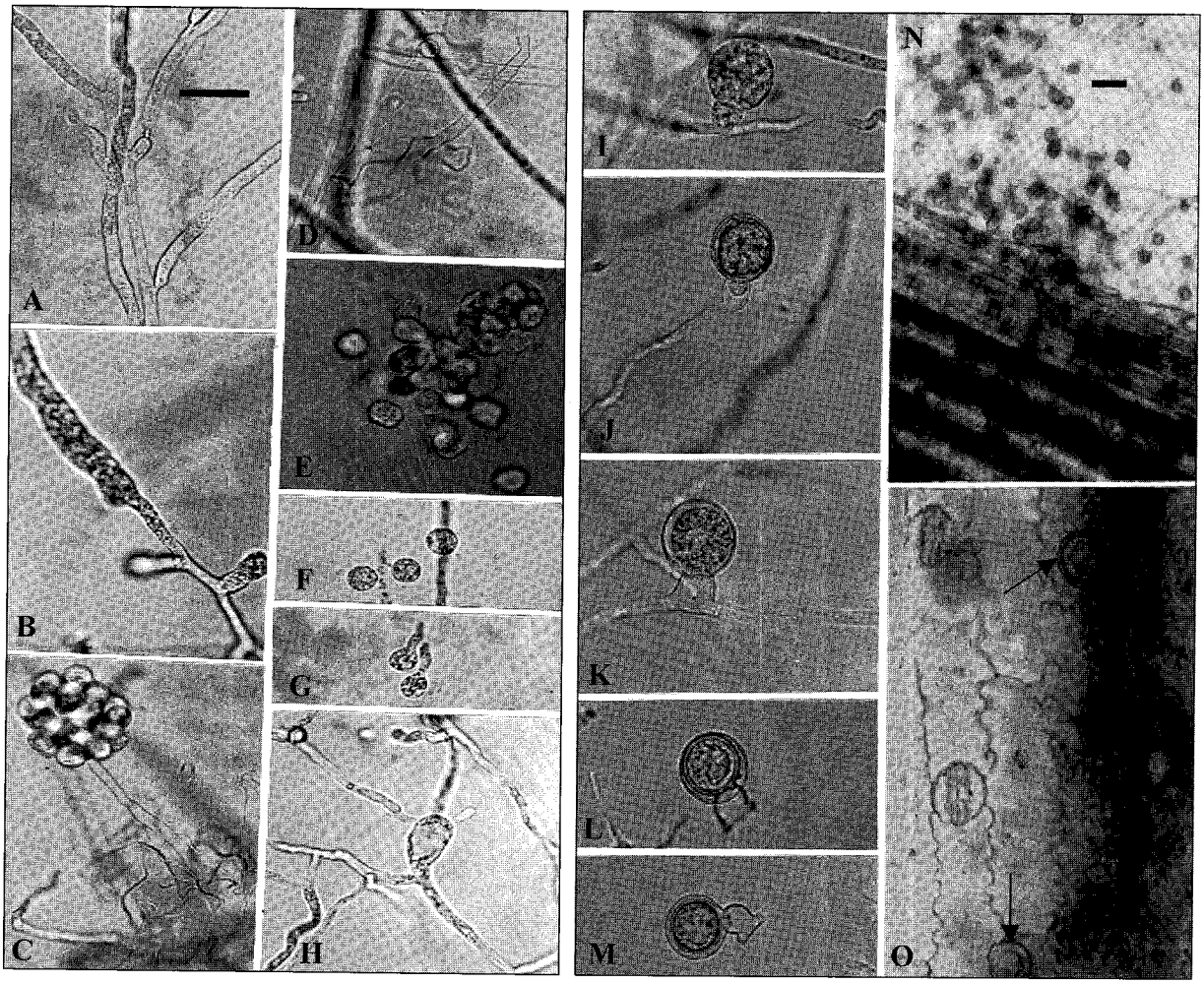

Fig. 1. Morphology of *Pythium aphanidermatum*. A. Mycelia. B. Young zoosporangium. C. A lobulate inflated zoosporangium, an evacuation tube and a vesicle containing zoospores. D. An empty inflated zoosporangium with evacuation tubes. E. Liberation of zoospores from a vesicle. F. Encysted zoospores. G. Germinating zoospores. H. An appressorium. I-O. Oogonia and intercalary antheridia. N. Many sexual structures originating from a leaf grass (low power). O. Oospores inside leaf cells (arrows). Bar (20 μm) on photo A is applicable to all photos but bar (40 μm) on photo N is only for this photo.

artificially infestated with *P. aphanidermatum* and mixed with *P. fluorescens*.

The emergence of bush okra was monitored visually for 50 days after sowing. For each treatment, the area used for plant counts corresponded to 300 per plot. All the results are the mean of 5 plots. The field experiment was performed in one season.

At the end of the experiment, superficial soil (upper 30 cm) of the plastic houses with artificial infestation by *P. aphanidermatum* was autoclaved.

Data were analysed according to Steel and Torrie (1980).

Results

Description of *Pythium aphanidermatum* (El-U522) isolated

***P. aphanidermatum* (Edson) Fitzp. (Fig 1, A-O)**

Colonies on CMA had a "cottony" aerial mycelium, but generally loose this aerial mycelium on PCA. Main hyphae were up to 11 μm wide. Zoosporangia consist of terminal complexes of swollen hyphal branches of varying length and up to 24 μm wide. Zoospores were formed at 15~30°C. Encysted zoospores had a diameter of 12 μm. Oogonia were terminal, globose, smooth and of (20-)22-25(-26) μm (av. 24 μm) diam. Antheridia were mostly intercalary, sometimes terminal, broadly sac-shaped, 11~ 15 μm long and 9~15 μm wide, 1(-2) per oogonium and monoclinous or diclinous. Oospores are aplerotic, (19-)20-24 μm (av. 22 μm) in diam and their walls are 1~2 μm thick.

Isolation of pathogens from rhizosphere soil and infected tissues of bush okra seedlings. Results of fungal isolations from the rhizosphere soil and from the infected bush okra seedlings indicated that isolates of *P. aphanidermatum* were the only parasites present.

Cardinal temperatures of *P. aphanidermatum*. Testing the effect of temperature on mycelial growth of *P. aphanidermatum* on CMA revealed that Minimum temperature for growth was below 10°C, optimum was 35°C, and maximum was above 43°C (Table 1).

Pathogenicity tests. *Pythium aphanidermatum* (Isolate 1, Isolate 2 and the reference isolate El-U520) were highly pathogenic causing 100% pre-emergence damping-off to germinating seeds of bush okra in the glasshouse when soil was amended with cornmeal-sand inoculum.

Post-emergence damping-off experiment indicated that *P. aphanidermatum* isolates (1, 2 and El-U520) were highly pathogenic to bush okra seedlings causing 95%, 93% and 88% post-emergence damping-off, respectively (Table 2, 3).

Phenotypic characteristics of *P. fluorescens* isolates. The isolates studied were non-sporeforming, motile, gram-negative and rod-shaped bacteria that grew on Mackonkey agar. The strains were identified based on production of fluorescent pigments on King medium and their denitrifying ability, growth at 4°C and lack of growth at 37°C (Table 4).

Table 2. Pre-emergence damping-off of bush okra (*Corchorus olitorius*) infected with *Pythium aphanidermatum* (Isolate 1, 2 and reference isolate No. El-U520) after 21 days of sowing in infested soil

No. of sown seeds	No. of emerged seeds	Pre-Emergence Damping-off (%)	Aphanidermatum isolate
250	250	0	Control*
250	0	100	Isolate 1
250	0	100	Isolate 2
250	0	100	El-U520†

*Non-infested soil.
†An isolate of *P. aphanidermatum* isolated from turnip rhizosphere in 2001 (Elnaghy *at al.*, 2002).

Table 3. Post-emergence damping-off of bush okra (*Corchorus olitorius*) infected with *Pythium aphanidermatum* (Isolate 1, 2 and reference isolate No. El-U520) 7 days after adding *P. aphanidermatum* inocula around the seedlings

No. of seedlings	No. of damped-off seedlings	Post-Emergence Damping-off (%)	Aphanidermatum isolate
214	0	0	Control[a]
208	198	95±2.3[b]	Isolate 1
204	190	93±1.8	Isolate 2
214	189	88±2.4	El-U520[c]

[a]Soil free of *Pythium*.
[b]Standard error of the mean.
[c]An isolate of *P. aphanidermatum* isolated from turnip rhizosphere in 2001 (Elnaghy *at al.*, 2002).
Each value represents the average of five measurements as indicated in post-emergence damping-off (%).

Table 1. Linear growth of *Pythium aphanidermatum* (Isolate 1, 2 and reference isolate No. El-U520) at various temperatures

P. aphanidermatum	Average growth (mm) after 24 h on corn meal agar									
	2°C	5°C	10°C	15°C	20°C	25°C	30°C	35°C	40°C	43°C
Isolate 1	0	0	5	14	26	35	47	51	39	5
Isolate 2	0	0	5	13	25	34	46	52	38	4
El-U520*	0	0	4	12	24	33	45	50	40	4

*Reference isolate of *P. aphanidermatum* from the first author's collection.

Table 4. Morphological, physiological, and biochemical properties of the isolated *Pseudomonas* spp.

Character	*P. fluorescens*	*P. aeruginosa*	*P. pudia*	*P. stutzeri*
Gram reaction	–	–	–	–
Spore formation	–	–	–	–
Motility	+	+	+	+
Growth at 4°C	+	–	–	–
Growth at 37°C	–	+	+	+
Growth in 7% NaCl	+	–	–	+
Production of fluorescent pigment	+	–	–	–
Growth on MacConky agar	+	+	–	–
Catalase	+	+	+	+
Oxidase	–	–	–	–
Urease	+	+	–	–
Nitrate reduction	–	–	+	+
Indole production	–	+	–	–
Citrate utilization	+	+	+	+
Starch hydrolysis	+	+	+	+
Gelatin hydrolysis	+	+	–	–
Casein hydrolysis	–	+	–	+
Lipid hydrolysis	+	–	+	+
Acid from glucose	+	+	+	+
Acid from fructose	+	+	+	+
Acid from sucrose	+	+	+	+
Acid from xylose	–	–	+	+
Acid from mannose	+	+	+	+
Acid from sorbitol	–	–	+	+
Penicillin test	+	+	–	–

Isolation of *P. fluorescens* strains from bush okra rhizosphere and non-rhizosphere soil. Fourty seven strains of *Pseudomonas* spp. which made inhibition zones against *P. phanidermatum* were isolated from rhizosphere of rotted bush okra seeds. Sixteen, 12, 10, and 9 strains of *P. fluorescens*, *P. aeruginosa*, *P. pudia*, and *P. stutzeri*, respectively, were isolated from bush okra rhizosphere soil by comparison with reference strains with a series of standard tests. Some morphological and physiological variations between strains were noted (Table 4). Using the method of Koike (1967), 16 strains of *P. fluorescens* were tested against *P. aphanidermatum* on agar plates in order to select those that were most antagonistic to *P. aphanidermatum* to be used for the biological control trial. Only seven strains isolated from bush okra non-rhizoshere soil were shown to be *P. fluorescens.*

Antagonism between *P. aphanidermatum* and *P. fluorescens* strains on agar plates. Sixteen strains of *P. fluorescens* together with the reference strain (DSM 1380)) were tested for their antagonism to *P. aphanidermatum* Isolates 1 and 2 by measuring the inhibition zone around each strain of *Pseudomonas fluorescens*. The most inhibitory bacterial [No. 1 = El-U444 and No. 2 = El-U445 (deposited at Botany Department, Faculty of Science, El-Minia University 61519, Egypt)] strains were selected to perform the biological control experiments (Table 5).

Effect of *P. fluorescens* on *P. aphanidermatum* damping-off in the pots. The two strains of *P. fluorescens* dramatically increased emergence of bush okra in infested soil with *P. aphanidermatum*. Soil treatment with bacteria was more effective than a seed coat. Plants grown in soil

Table 5. Effect of antagonism of 16 isolates of *Pseudomonas fluorescens* against three isolates of *Pythium aphanidermatum* on potato dextrose agar at 27°C

Pythium	Diameter of the inhibition zone around the bacterium (mm) *Pseudomonas fluorescens* isolates																
	RS[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Isolate 1	17	25	24	22	22	22	21	21	21	20	20	21	19	19	19	18	17
Isolate 2	17	26	24	22	22	21	21	22	20	20	21	19	19	20	19	17	16
El-U520[a]	18	25	23	22	22	23	21	21	22	21	22	21	21	20	20	18	18

[a]Reference isolate of *P. aphanidermatum* from the first authors collection.
[b](Reference strain, DSM 1380) Deutsche Samlung von Mikroorganismen, Brauenschweig, Germany.
[c]Each value represents the average of four measurements taken from two independent experiments.

Table 6. Effect of application of two strains of *Pseudomonas fluorescens* on bush okra (*Corchorus olitoris*) germinating seeds and seedlings grown in autoclaved and natural soil infested with two isolates of *Pythium aphanidermatum* + reference isolate (El-U520)

Treatment	Pre-emergence damping-off (%)		Post-emergence damping-off (%)	
	Autoclaved soil	Natural soil	Autoclaved soil	Natural soil
Control (bush okra only)[a]	0	0	0	0
Bush okra + *P. fluorescens*, strain 1 (soil mixing)[b]	0	0	0	0
Bush okra + *P. fluorescens*, strain 2 (soil mixing)[b]	0	0	0	0
Bush okra + *P. fluorescens*, strain 1 (seed coating)[c]	0	0	0	0
Bush okra + *P. fluorescens*, strain 2 (seed coating)[c]	0	0	0	0
P. aphanidermatum (Isolate 1)	100	98±1.3[d]	98±1.1	95±1.8
P. aphanidermatum (Isolate 2)	100	95±2.4	97±3.2	95±2.8
P. aphanidermatum (El-U520)	100	95±3.4	90±3.1	87±2.2
Pythium Isolate 1 + *P. fluorescens*, strain 1(soil mixing)	0	0	0	0
Pythium Isolate 1 + *P. fluorescens*, strain 2 (soil mixing)	1±0.1	0	0	0
Pythium Isolate 2 + *P. fluorescens*, strain 1 (soil mixing)	0	0	0	0
Pythium Isolate 2 + *P. fluorescens*, strain 2 (soil mixing)	2	0	0	0
Pythium Isolate 1 + *P. fluorescens*, strain 1 (seed coating)	0	0	ND[e]	ND
Pythium Isolate 1 + *P. fluorescens*, strain 2 (seed coating)	2±0.2	0	ND	ND
Pythium Isolate 2 + *P. fluorescens*, strain 1 (seed coating)	1±0.1	0	ND	ND
Pythium Isolate 2 + *P. fluorescens*, strain 2 (seed coating)	1	0	ND	ND
Pythium isolate El-U520 + *P. fluorescens*, strain 1 (soil mixing)	0	0	0	0
Pythium isolate El-U520 + *P. fluorescens*, strain 2 (soil mixing)	0	0	0	0
Pythium isolate El-U520 + *P. fluorescens*, strain 1 (seed coating)	0	0	ND	ND
Pythium isolate El-U520 + *P. fluorescens*, strain 2 (seed coating)	0	0	ND	ND

[a]Bush okra grown in non-infested soil.
[b]Bush okra grown in non-infested soil but the soil was mixed with *P. fluorescens*.
[c]Bush okra grown in non-infested soil but seeds were dressed with *P. fluorescens* by the aid of 2% carboxymethylcellulose.
[d]Standard error of the mean.
ND, not determined.
Each result is the mean of 5 replications as indicated by (pre-and post-emergence damping-off %).

not containing *Pythium* and treated with bacteria, either by seed coating or by adding bacteria to the soil, increased emergence (Table 6).

Effect of *P. fluorescens* on *P. aphanidermatum* damping-off in the plastic houses. Isolate 1 of *P. fluorescens* (El-U444) increased emergence (P = <0.05). Soil of plastic houses treated with the bacteria were more effective than a seed coat. Plastic houses which were naturally or artificially infested with *P. aphanidermatum* and treated with the bacteria by mixing the bacteria with the soil and sowing adhered bush okra seeds treated with the bacteria gave 100% plant emergence (Fig. 2).

Discussion

We identified *Pythium aphanidermatum* as the cause of bush okra damping-off in Der Attia village, El-Minia, Egypt for the first time in the world. In Egypt, there were no reports about fungal diseases of bush okra. The pathogenicity of *P. aphanidermatum* was proved (Elnaghy *et al.*, 2002; Plaats-Niterink, 1981). It can cause root rot and damping-off, stalk and rhizome rot, soft rot, fruit rot or cottony blight of *Abelmoschus esculentus*, *Basella* sp., *Carica papaya*, *Catharanthus* sp., *Carthamus* sp., *Chrysanthemum* sp., *Citrus* sp., conifers, corn, a number of other crops, crucifers, cucurbitaceae, leguminosae, *Fragaria* sp., *Gossypium* sp., grasses, *Ipomoea*, *Lactuca sativa*, *Linum usitatissimum*, peppers, poinsettia, *Solanum* sp., sugar beet, sugar cane, *Talinum* sp., *Tephrosia* sp., tobacco, tomato and *Viola* sp. (Plaats-Niterink, 1981). No reports were recorded conserning the pathogenicity of *P. aphanidermatum* to bush okra.

Temperature has a great influence on infection and damage caused by *P. aphanidermatum*. Temperatures of 30~35°C were most favourable for infection, while at lower temperatures it is less or negligible. In infected plastic houses, temperature reached 30°C which was favourable for infection (Plaats-Niterink, 1981).

The two isolates of *P. aphanidermatum* together with the reference isolate (isolated from the rhizosphere of turnip in 2001) proved to be highly pathogenic to germinating seeds and seedlings of bush okra. For edible leaf crops, chemical control can not be used to overcome this disease, however, some illegal application of fungicides were used with no effective results (personal communication).

Results presented in this paper highlight the ability of two *P. fluorescens* strains isolated from the rhizosphere of

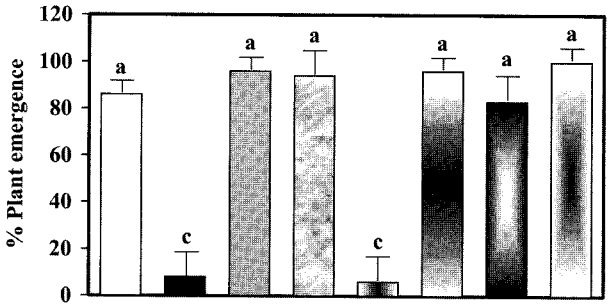

Fig. 2. Effect of application of *Pseudomonas fluorescens* (El-U444) on damping-off disease of bush okra (*Corchorus olitorius*) in clay-loamy soil infested with *Pythium aphanidermatum* after 50 days of sowing in 7 plastic houses (2 m × 6 m). Three control sets were used, ☐ bush okra in non-infested soil, ▩ bush okra in non-infested soil but with soil mixing by bacteria, and one ▩ as bush okra in non-infested soil but with seed coating by bacteria with the aid of 2% carboxymethyl-cellulose. Comparison of amendment of bacteria either by mixing them with soil ▩, or by coating bush okra seeds in bacterial suspension ▩ with the aid of 2% CMC, or by mixing bacteria with the soil and coating bush okra seed in bacterial suspension in infested soil by *P. aphanidermatum* ▩ was made. ■ The plastic house that naturally infestedd by *P. aphanidermatum*. ▩ The plastic house that artificially infested by *P. aphanidermatum*. Error bars represent standard deviations. Statistical differences between treatments are indicated with letters a, b and c.

bush okra to protect the plants against *P. aphanidermatum* in both pot and plastic house experiments. The two strains studied and the reference isolate of *P. fluorescens* significantly and reproducibly reduced *Pythium* damping-off in bush okra germinating seeds and seedlings. In the literature, the application of *P. fluorescens* to control diseases caused by *Pythium* spp. has been shown by several researchers (Abdelzaher *et al.*, 1998; Dunne *et al.*, 1998; Ongena *et al.*, 1999; Paulitz and Belanger, 2001; Weststeijn, 1990).

In Canadian green houses, *Pythium* is one of the most important root and seedling pathogens, on both vegetables and horticultural crops. In British Colombia, *P. aphanidermatum*, *P. irregulare*, *Pythium* sp. and *Pythium* "group G" were responsible for root disease and crown rot of greenhouse cucumber (Favrin *et al.*, 1988). In Quebec, *P. aphanidermatum* and *P. ultimum* were the species most commonly isolated from green house cucumber (Paulitz and Belanger, 2001). Many isolates of bacteria were isolated from the rhizosphere of cucumber (*Cucumis sativus* cv *Corona*) grown in 34 agriculture and forest soils collected in Quebec. Only 15% of the isolated bacterial strains inhibited mycelial growth. Most of these strains were *P. fluorescens* subgroups C and E, *P. corrugata* or *Pseudomonas* spp.. Furthermore, in a spring crop, *P. fluorescens* strain 15 produced 88% more marketable fruit than the inoculated control. Accordingly, strain 63~28 of *Pseudomonas fluorescens* has been sold to Ecosoil Inc., and EPA registration was submitted in November 1998 for a product called ATEZe, based on strain 63~28 that would be used as a soil drench for greenhouse vegetable crops to control *Pythium* and *Rhizoctonia*; (Paultiz and Belanger, 2001).

In this study, introducing *P. fluorescens* in *Pythium*-infested soil resulted in higher amounts of seedling emergence of bush okra. Soil amended with *P. fluorescens* resulted in better control of bush okra damping-off than seed coated with the bacteria. This may suggest an additional effect occurring outside rhizosphere, although the possibility of a better colonization of the rhizosphere and the roots cannot be excluded. The bacteria on the bush okra seed surface have to grow and spread to colonize the radicle and plumule when applied on the seeds. With soil amended with the bacteria a continuous supply of colonization of the roots from the bulk soil may be envisaged. The degree of germinating seed colonization, however, was not determined.

Ongena *et al.* (1999), pointed out that antifungal compounds induced by inoculation of cucumber root with two strains of fluorescent *Pseudomonas* participate actively in the protection of cucumber plants against *P. aphanidermatum*. Their results showed that inhibition zones (mm) caused by the antagonism of fluorescent *Pseudomonas* strains against *P. aphanidermatum* on agar medium were up to 5 mm. In our results *P. fluorescens* isolates, 1 and 2 showed 24 and 26 mm inhibition zones against *P. aphanidermatum*. Accordingly, strains 1 and 2 of *P. fluorescens* studied here produced antifungal compounds that inhibit the growth of *P. aphanidermatum* the causal agent of damping-off of bush okra.

Results from the plastic houses showed that, mixing bacteria with the soil and sowing treated bush okra seeds gave 100% emergence in infested soil by *P. aphanidermatum*. For this reason, application of bacteria with the two methods is the advisable way to get highest emergence. In conclusion, bush okra grown in plastic houses should be treated with *P. fluorescens* as seed dressing and mixing the bacteria with the soil before sowing. Steps are being taken toward registering the two isolates of *P. fluorescens* EL-444 and EL-445 as commercial biofungicides against *P. aphanidermatum*, the causal agent of damping-off of bush okra.

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