Interactions of Heterotrophic Bacteria from Recycled Greenhouse Irrigation Water with Plant Pathogenic *Pythium*

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Abstract. Heterotrophic bacteria present in recycled greenhouse irrigation water (RIW) were characterized and then evaluated for their effect on *Pythium aphanidermatum*, *P. cryptorregulare*, and *P. irregulare*. Nutrient agar (NA) and R2A agar were used to isolate copiotrophic and oligotrophic bacteria. Bacterial isolates recovered from RIW were categorized according to whether they inhibited *Pythium* growth, attached to hyphae, or enhanced *Pythium* growth in the three *Pythium* species used. Three bacterial isolates were selected to determine whether their in vitro interactions with *Pythium aphanidermatum*, the most pathogenic of the three species used, influenced disease development in the greenhouse. An isolate of *Sphingobium* sp. that inhibited *Pythium*, *Pseudomonas* sp. that attached to hyphae, and *Cupriavidus* sp. that enhanced the growth of *P. aphanidermatum* in vitro were used in greenhouse experiments to examine their effects on disease development in geranium (*Pelargonium × hortorum* ‘White Orbit’) grown in pasteurized potting mix in ebb and flood irrigation systems. Disease progress curves evaluating the effect of each bacterium indicate that they did not suppress or enhance disease development ($P = 0.05$). Thus, the effects that the bacterial isolates had in vitro differed from their effects under greenhouse conditions.

Recycling of irrigation water in commercial greenhouses is implemented as a strategy to minimize water and pesticide or fertilizer runoff from contaminating the environment (Hong and Moorman, 2005; Hu et al., 2008). However, recycling water increases the risk of accumulating and dispersing *Pythium* species that produce zoospores as well as other plant pathogens. This genus contains some phytopathogenic root pathogens that can cause severe losses in greenhouse crops, particularly those grown in soilless cultivation systems (Postma et al., 2005). Research that provides insights into the relationship among organisms of a microbial community in relation to *Pythium* spp. is important to build a better understanding of

Pythium species (Uzuhashi et al., 2010) and their interaction with plants.

The coexistence of bacterial isolates that inhibit or enhance the growth of *Pythium* should be considered when investigating an ecosystem. Naturally occurring microorganisms have the ability to suppress diseases (Berger et al., 1996; Van Os & Van Ginkel, 2001) even in soilless systems (Postma, 2009). Frequent coisolation of bacteria and *Pythium* and *Phytophthora* species suggests possible interspecies communication (Kong et al., 2010) and a close relationship among these groups of microbes and their ubiquitous coexistence may indicate a synchronous relationship with beneficial outcomes for one or both populations. Synergistic interaction studies between *Pythium* and coniche-inhabiting microbes are few. It is unknown to what extent bacteria may play a role in the survival and spread of *Pythium*. Closer attention should be given to the interaction and outcome of different microorganisms, especially the highly managed greenhouse environment.

One biocontrol mode of action against *Pythium* in soil is described as a general suppression phenomenon (Hoitink et al., 1997; Pane et al., 2013) as a result of a diverse group of microorganisms and weak competitiveness of *Pythium* (Ho, 1986), although

specific suppression phenomenon has also been described (Postma et al., 2009; Weller et al., 2002). In the aquatic environment, naturally occurring bacteria may play a role in the survival, suppression, or spread of *Pythium*. Therefore, the assessment of the microbial diversity in recycled irrigation water deserves attention. The stimulation of beneficial microorganisms known to survive in recycled irrigation water and able to suppress phytopathogenic *Pythium* species survival could be exploited as a treatment of recycled irrigation water in greenhouses.

The goal of this study was to identify *Pythium*-enhancing and *Pythium*-inhibiting bacteria residing in water being recycled in commercial greenhouses and determine if the in vitro effects of these bacteria on *Pythium* reflect their effects on disease development under greenhouse conditions.

Material and Methods

***Pythium* isolates.** Isolates of three phytopathogenic *Pythium* species frequently found in commercial greenhouse crops in the northeastern United States were used for this study, *Pythium aphanidermatum* (Edson) Fitzp., *P. cryptorregulare* (Garzón, Yáñez, and Moorman), and *P. irregulare* Buisman. Cultures were maintained on colonized water agar (WA) blocks in sterile tap water tubes stored at room temperature. The WA blocks were transferred to potato dextrose agar (PDA) in petri plates to start active cultures. Subcultures were transferred to WA or PDA and incubated at room temperature for 3 d to use as inoculum for further testing.

Recycled irrigation water collection. RIW samples collected every ≈60 d for 3 years (2009–12) from cement tanks (23,000-L tank in Greenhouse A; 76,000-L tank in Greenhouse B) located inside two commercial greenhouses in Pennsylvania were processed to isolate heterotrophic bacteria. In both greenhouses, well water is used to initially fill the tanks. The only treatment of the water following irrigation is coarse filtration to prevent large particulates from entering the tanks. Both greenhouses produce a wide variety of bedding plants as well as poinsettias each year. Water was collected in sterile 1-L glass jars, near the surface (less than 1 m depth) and from the bottom (greater than 2 m depth) using a device that kept the jars sealed until they were at the desired depth. The jars were placed in a cooler containing ice packs that were not in direct contact with the jars and were transported to the laboratory. The dissolved oxygen level was immediately measured (Traceable® Digital Oxygen by Control Company, Friendswood, TX) at the time of collection. Electrical conductivity (Model SD-B15; Beckman Industrial) and pH (Mettler-Toledo SevenEasy model, Columbus, OH) were measured after transport to the laboratory.

Heterotrophic bacteria from recycled water. Two media were used for isolation of bacteria, NA (Difco™ Nutrient Agar; Beckton, Dixon, and Co., Sparks, MD) for non-fastidious copiotrophic microorganisms and

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Fig. 1. Average colony-forming units (CFU) of copiotrophic bacteria on nutrient agar (NA) and oligotrophic bacteria on R2A isolated from recycled irrigation water in Greenhouse A (A) and Greenhouse B (B).

Fig. 2. Photos of three bacterial phenotypes. (A) Zone of inhibition of *Pythium aphanidermatum* by *Sphingobium* sp. (B) Microscopic view (10x) of wet appearing *P. irregulare* hyphae caused by the attachment of *Pseudomonas* sp. (C) *P. cryptoirregulare* growth enhancing effect by *Cupriavidus* sp. in NA media.

R2A (Difco™ R2A Agar; Beckton, Dixon, and Co.) for slow-growing oligotrophic microorganisms as follows. One milliliter of RIW was added to a glass tube containing 9 mL of sterile M9 (1× salt solution; 12.8 g Na₂HPO₄; 3 g KH₂PO₄; 0.5 mg NaCl; 1.0 g NH₄Cl, per 1 L of sterile water). Serial dilution up to 10⁻⁹ was used to determine colony-forming units per milliliter (CFU/mL) and to obtain individual, morphologically different colonies. Five hundred microliters from each dilution tube were transferred to NA and R2A media in petri plates and streaked with a sterile glass rod. NA plates were incubated at 23 °C for 3 d and R2A plates at 15 °C for 7 d. Morphologically different colonies were transferred to new media plates to obtain pure cultures. Each isolate was transferred to full-strength nutrient broth (NB; EMD Chemicals, Inc., Gibbstown, NJ) from NA plates and one-fourth strength NB from R2A plates and incubated at 23 and 15 °C, respectively. Fifty microliters from NB tubes were streaked on NA and R2A to confirm the purity of the culture. Pure cultures were transferred to NB tubes for future studies and for DNA extraction. Bacterial isolates were transferred to sterile tubes with silica gel granules (6 to 12 mesh, grade 40; Aqua Solutions, Deer Park, TX) for storage by mixing 1-week-old bacteria with 2.5 mL of sterile skim milk solution (10% skim milk and 15% glycerol) and applying this to the granules. These tubes were stored at -80 °C.

Pythium–bacterium in vitro interaction. To examine the in vitro effects of bacteria on *Pythium*, 50 µL of 48-h-old bacterial isolates from NB were placed on the margin of M9 minimum media agar (12.5 g Na₂HPO₄; 3 g

KH₂PO₄; 0.5 g NaCl; 1.0 g NH₄Cl; 1 M MgSO₄; 1 M MgSO₄, 2 mL of 20% glucose, per 1 L of sterile water) in petri plates and incubated at 21 °C in the dark. After 24 h, a *Pythium aphanidermatum*, *P. irregulare*, or *P. cryptoirregulare*-colonized WA plug (5 mm diameter) was placed on the opposite side of the plate containing the bacterium. Plates were incubated at 21 °C for 3 d and observed daily. A clear zone of growth inhibition was used as an indication of a deleterious effect on the development of *Pythium*. After 72 h, radii of the mycelial growth in the direction of the bacterium was measured and percentage of inhibition was calculated, $100 \times (R_1 - R_2)/R_1$, where R_1 is the maximum radius of *P. aphanidermatum*, *P. cryptoirregulare*, or *P. irregulare* colonies in bacteria-free control plates and R_2 is the radius of the colonies on the plates containing bacteria (Paulitz et al., 1992). A few bacterial isolates enhanced *Pythium* growth in vitro when compared with control plates (*Pythium* only). Enhancement of *Pythium* growth by bacterial isolates was calculated using a modified version of the equation above $100 \times (R_2 - R_1)/R_2$. In addition, bacterial isolates that attached to and grew extensively on *Pythium* hyphae in media plates were isolated and kept for identification. Bacterial isolates that attached to *Pythium* grew next to the hyphae giving cultures a wet appearance in vitro. This wet growth was used as an initial indication of the bacterium–hyphal attachment phenotype, which was then confirmed using light microscopy and scanning electron microscopy (SEM). This attachment has been associated with a growth reduction of *P. ultimum*

(Nelson et al., 1986). In our study this phenotype was associated with a tendency to slow *Pythium* hyphal growth when compared with a control (*Pythium* only), but the inhibition was not statistically significant. A total of 900 isolates were tested in vitro. All bacterial isolates were tested at least three times and only isolates with consistent results were kept for identification.

Bacterium–hyphal attachment. One bacterium isolate (756[^]-2 1.0 N.2 identified as *Enterobacter* sp.) was used as representative of the group of bacterial isolates identified as attaching to *Pythium* hyphae. *Enterobacter* sp. was incubated with *P. aphanidermatum* hyphae to verify attachment. Two samples were prepared under different conditions. One sample was incubated on solid medium agar containing glucose. In this method, a solid surface was needed on the medium to obtain an *Enterobacter*–*P. aphanidermatum* hyphal sample that could be further processed for SEM. A sterile crepe filter paper (VWR Grade 615, West Chester, PA) was placed on the center of a 2% glucose M9 agar plate (100 × 65 mm). One hundred microliters of a 24-h *Enterobacter* sp. was added to the medium around the edge of the filter. This plate was incubated at 25 °C for 24 h at which time three 3-mm diameter WA plugs of a 24-h *P. aphanidermatum* were transferred to the edge of the filter on the M9 agar plate and incubated at 25 °C for 3 d. The filter was used to prepare the sample for SEM. In the second method used, a 24-h, 3-mm diameter WA plug of *P. aphanidermatum* was transferred to 20% V8 broth (V8 vegetable juice; Campbell Soup Co., Camden, NJ) for 3 d. After 3 d, the hyphal mat was transferred to a 24-h *Enterobacter* sp. culture grown on 9 mL NB (1/100 dilution) and incubated at 25 °C for 3 d. After 3 d, all specimens were prepared for SEM to confirm the attachment of bacteria to *Pythium* hyphae.

SEM sample preparation. Briefly, samples were prepared according to Dykstra (1992). Samples were fixed overnight with 2.5% glutaraldehyde, washed three times with 0.1 M phosphate buffer, and dehydrated in graded ethanol series (25%, 50%, 70%, 85%, 95%, and 100%) for 5 min at each step. After dehydration, the samples were critical point-dried and coated with gold before being examined in a scanning electron microscope (JSM 5400, Peabody, MA).

Heterotrophic bacteria identification. To extract DNA, bacteria were grown in NB tubes and incubated at 21 °C for 48 h and a modified thermal lysis DNA extraction method (Queipo-Ortuno et al., 2008) was used. Briefly, 1 mL of bacterium culture in NB was transferred to a 2-mL heat-resistant microcentrifuge tube and centrifuged (Eppendorf Model 5415C) at 15,000 g for 15 min. The supernatant was discarded and the pellet was resuspended in 50 µL of sterile distilled water (SDW). Tubes were transferred to a heating block at 99 °C for 10 min, cooled on ice for 2 min, and centrifuged at 15,000 g for 1 min. Supernatant was transferred into a new tube and labeled. The DNA concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo

Table 1. Identity and impact of bacterial isolates from recycled greenhouse irrigation water on three species of *Pythium*.

Strains ^z	Bacterial genus	Genus sequence ID (%) ^y	<i>Pythium aphanidermatum</i>		<i>Pythium irregulare</i>		<i>Pythium cryptorregulare</i>	
			Percentage ± SE (%) ^x	Association ^w	Percentage ± SE (%)	Association	Percentage ± SE (%)	Association
432 ^Δ -2 1.0 NA*	<i>Acidovorax</i> sp.	99	0	N	3.9 ± 0.7	I	0.9 ± 0.3	A
746 1.0 N.4	<i>Acinetobacter</i> sp.	98	0	N	0.1 ± 0.01	A	0	N
750 ^Δ -1 R.1	<i>Acinetobacter</i> sp.	98	1 ± 0.4	A	0.9 ± 0.3	A	0.1 ± 0.01	A
747 ^Δ -3 N.4	<i>Acinetobacter</i> sp.	99	0.1 ± 0.01	A	0	N	0	N
756 ^Δ -2 1.0 N.2 ^u	<i>Enterobacter</i> sp.	100	1 ± 0.4	A	0.1 ± 0.01	A	0.8 ± 0.2	A
756 ^Δ -2 1.0 N.3	<i>Enterobacter</i> sp.	99	0.6 ± 0.2	A	0.9 ± 0.3	A	0.7 ± 0.2	A
756 ^Δ -4 1.0 R.2*	<i>Enterobacter</i> sp.	99	0.9 ± 0.3	A	20 ± 2.1	I	7.8 ± 0.8	I
759 ^Δ -7 R.2A	<i>Pantoea</i> sp.	99	0.3 ± 0.1	A	0	N	0	N
439 ^Δ -2 R.3*	<i>Pseudomonas</i> sp.	100	0.3 ± 0.1	A	13.3 ± 0.8	I	0	N
439 ^Δ -3 R.1*	<i>Pseudomonas</i> sp.	100	0.5 ± 0.2	A	8.4 ± 0.6	I	7.7 ± 1.3	I
432 1.0 N.2*	<i>Pseudomonas</i> sp.	97	17.2 ± 2	I	0.2 ± 0.02	A	12.8 ± 1.3	I
746 ^Δ -3 R.4	<i>Pseudomonas</i> sp.	99	0.5 ± 0.2	A	0	N	0	N
748 ^Δ -2 1.0 N.4	<i>Pseudomonas</i> sp.	99	0.5 ± 0.2	A	0	N	0	N
748 ^Δ -1 N.1*	<i>Pseudomonas</i> sp.	100	0.4 ± 0.1	A	16.6 ± 1.0	I	0	N
438 ^Δ -7 R.1*	<i>Pseudomonas</i> sp.	99	0	A	1.7 ± 0.5	I	1.6 ± 0.8	I
438 ^Δ -3 1.0 N.1 ^v	<i>Pseudomonas</i> sp.	99	0.1 ± 0.01	A	0	N	0	N
751 ^Δ -3 R.2A	<i>Pseudomonas</i> sp.	97	0.9 ± 0.3	A	0.9 ± 0.3	A	0	N
431 ^Δ -2 N.1	<i>Pseudomonas</i> sp.	99	0.4 ± 0.1	A	0	N	0	N
434 ^Δ -2 N.3	<i>Pseudomonas</i> sp.	99	0.7 ± 0.2	A	0.8 ± 0.2	A	0.9 ± 0.3	A
752-3 1.0 R.3	<i>Sphingobium</i> sp.	99	32.6 ± 6	E	36.5 ± 2.8	E	7.1 ± 1.2	E
431 1.0 N.2	<i>Bosea</i> sp.	99	0	N	13.9 ± 1.1	E	5.8 ± 1.2	E
752-4 N.1	<i>Cupriavidus</i> sp.	99	0	N	0	N	6.7 ± 1.0	E
756-3 1.0 N.3 ^v	<i>Cupriavidus</i> sp.	99	26.2 ± 3	E	0	N	0	N
752-4 1.0 R.3	<i>Cupriavidus</i> sp.	99	16.2 ± 3	E	19.3 ± 1.5	E	0	N
755 1.0 NA	<i>Herminiimonas</i> sp.	99	0	N	18.6 ± 1.1	E	34.6 ± 4.9	E
440-2 N.2	<i>Pseudomonas</i> sp.	99	11.3 ± 2	E	0	N	14.2 ± 1.7	E
436-5 1.0 R.3	<i>Pseudomonas</i> sp.	96	15.8 ± 3	E	0	N	4.7 ± 0.7	E
752-2 1.0 N.1	<i>Pedobacter</i> sp.	97	22.3 ± 2	E	8.7 ± 0.9	E	0	N
435 ^Δ -6 1.0 R.2	<i>Mycobacterium</i> sp.	96	10.8 ± 2	E	0	N	12.5 ± 2.2	E
437-5 1.0 R.1	<i>Mycobacterium</i> sp.	96	15.5 ± 3	E	0	N	0	N
439 ^Δ -1 NA	<i>Bacillus</i> sp.	99	4.1 ± 0.9	E	0	N	12.1 ± 1.3	E
431 ^Δ -1 1.0 N.1	<i>Pseudomonas</i> sp.	100	11.2 ± 1	I	8.5 ± 0.6	I	8.9 ± 1.2	I
433 ^Δ -1 1.0 N.2	<i>Pseudomonas</i> sp.	99	9.8 ± 1.1	I	5.9 ± 0.6	I	11 ± 1.5	I
750 ^Δ -1 N.1*	<i>Pseudomonas</i> sp.	99	0.2 ± 0.02	A	20.7 ± 1.3	I	8.6 ± 0.8	I
749 ^Δ -1 N.5	<i>Pseudomonas</i> sp.	99	0	N	29 ± 1.7	I	0	N
437 ^Δ -3 1.0 R.1	<i>Pseudomonas</i> sp.	100	0	N	2.1 ± 0.6	I	3.7 ± 0.7	I
433 1.0 N.4	<i>Pseudomonas</i> sp.	99	7.7 ± 0.8	I	0	N	9.4 ± 1.3	I
749 ^Δ -1 R.4*	<i>Pseudomonas</i> sp.	100	0.4 ± 0.1	A	8.3 ± 1.2	I	2.9 ± 0.6	I
435 ^Δ -6 1.0 R.3	<i>Pseudomonas</i> sp.	99	0	N	2.7 ± 0.6	I	0	N
747 ^Δ -2 R.3	<i>Pseudomonas</i> sp.	98	0	N	15.2 ± 2.4	I	0	N
431 1.0 N.1 ^v	<i>Sphingobium</i> sp.	99	8.6 ± 1.2	I	8.5 ± 0.6	I	8.9 ± 0.6	I
440 ^Δ -2 R.3	<i>Sphingobacterium</i> sp.	99	0	N	6.1 ± 0.6	I	0	N
439 ^Δ -2 N.4	<i>Chryseobacterium</i> sp.	99	0	N	5.6 ± 0.5	I	0	N
434 1.0 N.2	<i>Microbacterium</i> sp.	99	0	N	15.3 ± 2.4	I	4.6 ± 0.7	I
760 ^Δ -4 NA	<i>Bacillus</i> sp.	99	14 ± 2	I	0	N	4.4 ± 0.7	I
754 1.0 R.3	<i>Bacillus</i> sp.	99	0	N	6.9 ± 0.7	I	2.7 ± 0.6	I
439 ^Δ -2 N.6*	<i>Bacillus</i> sp.	99	6.3 ± 1.1	E	5.4 ± 0.6	I	16.3 ± 2.5	I
440 ^Δ -4 R.3	<i>Bacillus</i> sp.	100	0	N	6.1 ± 0.6	I	0	N

^zWater sample identification number. Bacterial isolates marked with * exhibited more than one type of in vitro effect.^yPercentage 16S rDNA sequence identity similarity.^xPercentage of growth inhibition of *Pythium* was calculated using equation (Paulitz et al., 1992), $100 \times (R_1 - R_2)/R_1$, where R_1 is the maximum radius of *Pythium* colonies in bacteria-free control plates and R_2 is the radius of the colonies on the plates containing bacteria. Percentage of *Pythium* growth enhancement by bacterial isolates was calculated using growth assessment equation (Paulitz et al., 1992) with modifications, $100 \times (R_2 - R_1)/R_2$ where R_2 is the maximum radii of *Pythium* colonies in bacteria-inoculated plates and R_1 is the radii of *Pythium* colonies in bacteria-free control plates. N = no enhancement, inhibition, or attachment to *Pythium*.^wResults according to in vitro phenotype: (I) inhibition, (A) attachment, (E) enhancement of *Pythium* spp. using growth assessment equation (Paulitz et al., 1992) and N = no reaction.^vUsed in greenhouse experiments.^uBacterium isolate used on scanning electron microscopy photograph as representative of the group attaching to *Pythium* hyphae.

Fisher Scientific, Waltham, MA) adjusted to 25 ng·μL⁻¹ with SDW, and stored at -20 °C until further use.

The polymerase chain reaction (PCR) technique was used to amplify the coding region of the 16S rDNA gene of bacteria with semi-universal primers EUB933 (5'-GCACAA GCGGTGGAGCATGTGG-3') and EUBr1387 (5'-GCCCGGGAACGTATTACCG-3') (Iwamoto et al., 2000). PCR master mix (Promega, Madison, WI) was used to carry out all the reactions as follow: 5 μL of PCR

(10×) standard *Taq* buffer, 1 μL of dNTPs (10 mM), 5 μL of each (10 μM) EUB 933/ EUBr1387 primer, 0.1 μL (1.25U) of *Taq* polymerase, 31.9 μL of SDW, and 2 μL of DNA template (25 ng·μL⁻¹) for a 50-μL PCR reaction. PCR cycles were carried out in a PTC-100™ or PTC-200™ Programmable Thermal Controller (MJ Research, Waltham, MA) as follows: 95 °C, 3 min; 10 cycles of 94 °C for 30 s, 68 °C for 45 s, decreasing 1.0 °C per cycle, 72 °C for 1 min; followed by 25 cycles of 95 °C for 30 s, 58 °C for 45 s, 72 °C

for 1 min; finishing with an extension step of 72 °C for 5 min. Electrophoresis was done on 2 μL of (6×) EZ-Vision™ Three, DNA dye Loading buffer (AMRESCO, Solon, OH) mixed with 3 μL of PCR product and loaded onto 1% agarose gel. Bands in the agarose gel were visualized in a ultraviolet transilluminator (256 to 366 nm) (UVP, LLC, Upland, CA).

PCR products were cleaned using Exo-SAP (New England Biolabs, Ipswich, MA) following the manufacturer's instructions

Table 2. Average measurements (n = 40) of recycled irrigation water (RIW) samples collected from two commercial greenhouses in Pennsylvania and used in this study.

Description ^z	Chemical properties ^y		
	pH	Dissolved oxygen (mg O ₂ /L)	Electrical conductivity (mS·cm ⁻¹)
Mean	6.16	4.13	1.49
Maximum	7.8	6.5	2.9
Minimum	5.3	2.0	0.3
SD	± 0.447	± 1.05	± 0.40

^zResults are for all samples from both greenhouses sampled.

^yDissolved oxygen measurements were taken on site at the time of sampling. Electrical conductivity and pH measurements were taken in the laboratory.

Fig. 3. Diversity and number of bacterial isolates from recycled irrigation water that inhibit, attach to, and enhance growth of *Pythium* spp. Identification was based on 16S rDNA sequences compared to sequences in GenBank database and Seqmatch from Ribosomal Database Project (Cole et al., 2009a).

and the DNA template was adjusted to 20 ng·μL⁻¹ for sequencing. The same primers used for the initial PCR reaction were used for DNA sequencing. Briefly, 2 μL of EUB f933/EUBr1387 (1×) and 2 μL of DNA template were transferred to a 96-well ultraAmpTM PCR plate. DNA sequencing was conducted in an ABI Hitachi 3730XL DNA analyzer at The Pennsylvania State University Genomic Core Facility, University Park, PA. Bacterial sequences from the forward and reverse primers were aligned using Sequencher 5.0 (Gene Codes, Ann Harbor, MI) to construct a contig of the sequence for each bacteria isolate. DNA contigs were compared with 16S rDNA gene sequences available using nucleotide BLAST from the National Center for Biotechnology Information GenBank and Seqmatch from the Ribosomal Database Project to identify the bacteria to genus (Cole et al., 2009).

Bacterial impacts on geranium stem rot in ebb and flood systems. To examine the effects of the bacteria on disease development, ebb and flood experimental units (American Hydroponics, Arcata, CA) were set up in a greenhouse at University Park, PA. One isolate, each representing a phenotype, *Sphingobium* (431 1.0 N.1; inhibited *Pythium* growth in vitro), *Pseudomonas* (438^Δ-3 1.0 N.1; attached to *Pythium* in vitro), and *Cupriavidus* (756^Δ-3 1.0 N.3; enhanced *Pythium* growth in vitro), were tested against *P. aphanidermatum*. Seeds

of geranium (*Pelargonium ×hortorum* cv. White Orbit) were planted in pasteurized sphagnum peat/perlite potting mix (Fafard #2; Conrad Fafard, Inc., Agawam, MA) in plastic seedling trays (200 cells/tray, 10 mL/cell) and grown in the laboratory for 5 d at 26 °C. The seedlings were then transferred to the greenhouse to acclimate. After 48 h, seedlings were transplanted to individual round, plastic pots (10 cm diameter × 8.5 cm) containing ≈400 mL of pasteurized Fafard #2 potting mix. Each experimental unit consisted of 16 pots (four rows of four pots) in a 1 m × 1-m plastic ebb and flood bench. Each bench had its own 30-L water reservoir and pump. Tap water containing soluble fertilizer (15% N–16% P₂O₅–17% K₂O; Peters Professional; The Scotts Company LLC, Marysville, OH) filled each reservoir. After 1 week, selected unit reservoirs were inoculated with a 48-h-old bacterium culture (10⁵ CFU/mL, final concentration). Each bacterium was cultured in 10 Erlenmeyer flasks (250 mL). One milliliter of a 24-h-old NB bacterium culture was added to 100 mL NB and incubated at 23 °C. After 48 h, the broth was centrifuged at 5000 g for 3 min. Bacterial pellets were diluted into 15 mL of M9 minimum media and use to inoculate ebb and flood systems. Five hundred microliters of the 15 mL broth was plated into M9 to quantify bacterial inoculum (10⁶ to 10⁷). The next week, units were inoculated with a 5- or 7-d-old *P. aphanidermatum* culture applied to the surface of the potting mix close to the stem (stem

inoculum) or directly into the water reservoir (water inoculum), respectively. *Pythium aphanidermatum*, the most pathogenic of the three species, was grown at 23 °C on NARF (25 mg nystatin, 150 mg ampicillin, 5 mg rifampicin, 5 μL fluazinam; 500 mL of 20% clarified V8 juice agar) (Morita and Tojo, 2007) plates for stem inoculations and 25 mL of 20% clarified V8 juice for water inoculations. The contents of one 60-mm diameter plate of colonized NARF agar was mixed using a blender (Waring Commercial, Torrington, CT) with 100 mL of sterile water to obtain *Pythium* stem inoculum. For *Pythium* water inoculum, V8 broth plates were washed three times with SDW and the *Pythium* mat was added to the water reservoir. We used experimental units with bacterium-only and non-treated units as controls. When *Pythium* was applied to plants (stem inoculum) in pots, eight plants were inoculated. Electronic timers controlled the pumps such that they ran for ≈10 min at each irrigation. An outflow pipe connected to the reservoir allowed the water to reach a depth of ≈2 cm in the ebb and flood tray before it began to drain back into the reservoir. The reservoirs were adjusted to 30 L with fertilizer-containing water and kept at that volume for the duration of the experiment. To monitor the presence of *Pythium*, all reservoirs were baited with blades of creeping bentgrass (*Agrostis stolonifera* L. ‘Penn Eagle’) that had been grown in the laboratory (Watanabe et al., 2008). Grass blades cut into sections were sandwiched between pieces of fiberglass window screen and suspended in the water. The blades were changed weekly in all systems. Blades were taken to the laboratory and individually placed on NARF agar for detection of *Pythium*. *Pythium* growing from the blades was transferred to water agar and identified weekly to verify the survival of *P. aphanidermatum*. In addition, to monitor the amount of bacteria, 5 mL of water was extracted from each system weekly and plated in NA media.

Greenhouse experimental units were arranged in a randomized complete block design. Each treatment was replicated twice with each replicate consisting of 16 geranium plants. The experiment was done from Jan. to Mar. 2012 and repeated from Apr. to June 2012. The number of geraniums infected per week was recorded based on water-soaked lesions on stems for each experimental unit. Disease incidence (DI) percentage was calculated based on the number of plants infected/total number of plants × 100 per experimental unit. The reduction of DI, as a measurement of effectiveness of the treatment in suppressing the

Fig. 4. Scanning electron micrographs of *Pythium aphanidermatum* and *Enterobacter* sp. from two specimen preparations. A) Specimen incubated over crepe filter paper on 2% M9 agar for 3 d, and B) and C) Specimens incubated on nutrient broth for 3 d.

Fig. 5. Mean area under the progress curve (AUDPC) of geranium stem rot in ebb and flow systems infested by adding mycelium mat of *Pythium aphanidermatum* to either potting mix [P(S)] or water reservoir [P(W)]. Bacterial species and strains: *Sphingobium* sp., 431 1.0 N.1 (Bac 1), *Pseudomonas* sp., 438⁺-3 1.0 N.1 (Bac 2), *Cupriavidus* sp., and 756⁺-3 1.0 N.3 (Bac 3), were added alone or in combination to a reservoir to examine antagonistic activities. Recycled irrigated water (RIW) and autoclaved recycled irrigated water (A-RIW) representing the presence and absence of total bacterial community also were included with P(W) for comparison purposes. Error bars represent the standard error of the mean. The graph shows the mean AUDPC of two independently performed experiments. Bars showing same letter are not significantly different (Kruskal-Wallis test, $P = 0.05$).

disease progress, was assessed using data plotted as area under the disease progress curve (AUDPC). AUDPC were calculated with the trapezoidal equation (Madden et al., 2007) using DI over time. In this method, DI data are collected over time. The average DI at the midpoint between two points is calculated by multiplying the average by the length of time between two points and summing the products across all time intervals.

Data analysis. Heterotrophic bacteria counts were log-transformed before analysis. The AUDPC data did not meet normal distribution, even after transformation with the arcsine square root. Therefore, the data were analyzed with Kruskal-Wallis test ($P = 0.05$) using XLSTAT 2013 (Addinsoft, France). This method is a nonparametric approach for

comparing data similar to analysis of variance (Sokal and Rohlf, 2012).

Results

Quantity of heterotrophic bacteria in greenhouse water reservoir. Two media were used to isolate heterotrophic fast-growing, non-fastidious copiotrophic bacteria and slow-growing oligotrophic bacteria from recycled irrigation water collected at two different commercial greenhouses.

The quantities of heterotrophic bacteria (CFU/mL) differed between greenhouses (Fig. 1). Usually, bacterial numbers were higher in the fall in Greenhouse A and in the spring in Greenhouse B coinciding with higher crop production in the respective greenhouses. Overall, Greenhouse A had higher numbers

of heterotrophic bacteria in 60% of the water samples (Fig. 1). The quantities of copiotrophic and oligotrophic bacteria differed. Water samples from Greenhouse A had 60% more CFU/mL of copiotrophic bacteria, whereas 50% of the samples had more oligotrophic bacteria.

Many morphologically different bacteria colonies were isolated from the RIW samples and ≈ 900 bacterial isolates were tested for their in vitro effect on the hyphae of the three *Pythium* species (Fig. 2). Whereas only 5% of the isolates exhibited inhibition, attachment to, or enhancement of *Pythium* growth (Table 1), they were isolated repeatedly from the greenhouses. The pH, electrical conductivity, and dissolved oxygen content of the water collected from two commercial greenhouses (Table 2) are within ranges that maintain bacterial growth.

Pythium-bacterium in vitro interaction. Twenty-four bacterial isolates inhibited the growth of at least one of the *Pythium* species (Table 1). The group was dominated by Gram-negative bacteria *Proteobacteria* and *Bacteroidetes*, whereas few isolates were Gram-positive *Actinobacteria* and *Firmicutes* (Fig. 3). Among the antagonistic bacteria, 14 were identified as *Pseudomonas*, four as *Bacillus*, and one isolate of each as *Microbacterium*, *Sphingobacterium*, *Enterobacter*, *Sphingobium*, or *Acidovorax*. We isolated bacteria that inhibited only one species, *P. irregulare*, *P. cryptoirregulare*, or *P. aphanidermatum*. Some bacterial isolates exhibited multiple effects, attaching to one *Pythium* species while inhibiting the growth of another *Pythium* species.

Twenty-one bacterial isolates, dominated by *Proteobacteria* (Fig. 3), attached to *Pythium* hyphae in vitro (Table 1). Most of the bacterial isolates that attached to *Pythium* hyphae decreased the growth of *Pythium* when compared with control plates, although the inhibition was not significant. Most of the isolates identified as *Pseudomonas* spp. specifically attached to *P. aphanidermatum* hyphae. Some bacterial isolates had multiple effects (attached to or inhibited *Pythium*) depending on the *Pythium* species. *Acinetobacter* spp.

isolates were closely related but not identical to one another. That was also the case for *Enterobacter* spp. *Pseudomonas* spp. isolates were very diverse. SEM of the *P. aphanidermatum*-*Enterobacter* sp. interaction confirmed that bacterial cells actually attached to *P. aphanidermatum* hyphae (Fig. 4). In Figure 4A, an intact hyphae can be seen with bacterial cells attached to the surface. In Figures 4B and 4C, *P. aphanidermatum* hyphae can be seen with bacterial cells aggregated near to a lysed hyphae. A similar association was observed with *P. irregulare* and *Enterobacter* sp.

Thirteen bacterial isolates enhanced the growth of *Pythium* in vitro (Table 1). Most were Gram-negative (*Proteobacteria* and *Bacteroidetes*) and a few were Gram-positive bacteria (*Firmicutes* and *Actinobacteria*) (Fig. 3). *Pythium aphanidermatum* growth was enhanced by 77% of the isolates, 62% enhanced the growth of *P. cryptoirregulare*, whereas 38% enhanced the growth of *P. irregulare*. One bacterium, identified as *Bacillus* sp., enhanced the growth of *P. aphanidermatum* but inhibited the growth of *P. cryptoirregulare* and *P. irregulare*.

Area under the disease progress curve. Disease symptoms caused by *P. aphanidermatum* developed 2 weeks after inoculating the units with the pathogen. Initial symptoms included stunting and water-soaked lesions on stems close to the soil line. Lesions on the stem progressed and eventually killed the plants. The systems with the least mortality (less than 40%) were those in which *Pythium* was applied to the potting mix (stem inoculum) and no bacteria had been added. *P. aphanidermatum* was detected in the units from the time of inoculation until 1 week after the plants were dead. Bacteria quantities were between 10^4 and 10^6 CFU/mL in all experimental units throughout the experiment. AUDPC value was highest in the experimental unit with *Pseudomonas* and *P. aphanidermatum* added to water and in the unit with all three bacteria and *P. aphanidermatum* in water (Fig. 5), but not different from most of the experimental units. None of the bacteria suppressed *Pythium aphanidermatum* that caused disease in geraniums under greenhouse conditions.

Discussion

The RIW from two commercial greenhouses was sampled over a 2-year period to identify those bacterial isolates with in vitro effects on *Pythium aphanidermatum*, *P. cryptoirregulare*, or *P. irregulare*. Water from both greenhouses had up to 10^9 CFU/mL of bacteria. Although two different media were used, one that favored copiotrophic bacteria and the other oligotrophic bacteria, most of the genera identified were isolated on both media. It is known that some genera can switch from copiotrophic to oligotrophic growth depending on the environmental conditions (Lauro et al., 2009). Microbial growth in nature is often characterized as "nutrient-limited" (Schmidt and Konopka, 2009), which explains

the mechanism of some microorganisms switching, depending on nutrient availability in the environment, and may explain why we isolated the same genera from both media. Although the two commercial greenhouses are geographically separated and have different sources of irrigation water, they harbored similar bacteria phyla in the water that was being recycled during crop production.

Among the over 900 selected heterotrophic bacteria evaluated, only a small group was identified as having in vitro effects on the growth of any of the three plant pathogenic *Pythium* species. Previous studies of the major phyla known to suppress *Pythium* spp. include *Pseudomonas* (*Gammaproteobacteria*) (Chen et al., 2012; Gardener, 2007), *Enterobacter* (*Gammaproteobacteria*) (Chen et al., 2012; Martin and Loper, 1999), and *Bacillus* (*Firmicutes*) (Chen et al., 2012). In this study, most of the heterotrophic bacteria isolated with antagonistic properties were identified as *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. The bacteria genera isolated in this study have been identified as containing species that act as biocontrol agents (Brooks et al., 1994; Nelson et al., 1986; Silva et al., 2012) against *Pythium* (Deniel et al., 2004; Mavrodi et al., 2012). The most abundant genus was *Pseudomonas*, which is known for its root-colonizing ability (Paulitz et al., 1992) and for its antagonistic properties against *Pythium* (Chen et al., 1998, 2012; Gardener, 2007). It was not surprising to find *Pseudomonas* species because they are widespread in the environment (Valerio et al., 2010), including aquatic ecosystems. Furthermore, these results suggest that *Pseudomonas* and other bacteria survived well under greenhouse conditions.

We identified some bacterial isolates that were able to attach to *Pythium* hyphae, mainly *P. aphanidermatum*, and exhibited a tendency to slow their growth in vitro, although the inhibition was not significant. This phenomenon has received sporadic attention (Leben, 1984; Siala and Gray, 1974; Wong and Griffin, 1976). Previous studies mention the ability of bacteria to attach to fungi (Nguyen Duc et al., 2011; Scheublin et al., 2010) and *Pythium* hyphae and inhibit their growth (Nelson et al., 1986). We observed the same association because *Pythium* grew slower in the presence of attached bacteria. Recently, a newly identified root-colonizing bacterium was found to attach to *P. aphanidermatum* hyphae infecting cucumber seed (Ofek et al., 2012). It is not clear what this relationship represents but the specificity to *P. aphanidermatum*, a highly phytopathogenic species, by the bacterium deserves further investigation.

A surprising finding was the identification of bacterial isolates that stimulate the growth of the three *Pythium* isolates. To our knowledge, this is the first report of this phenomenon. It is not known to what extent bacterial isolates enhance the survival of *P. aphanidermatum*, *P. cryptoirregulare*, and *P. irregulare* in RIW reservoirs, but this should be explored. We have no evidence of why these organisms

enhanced the growth of *Pythium* in vitro or if this occurs under natural conditions.

A few bacterial isolates exhibited different interactions with the different *Pythium* species. In the present research, *Bacillus* was identified for its in vitro ability to enhance the growth of *P. aphanidermatum* and inhibit growth of *P. irregulare* and *P. cryptoirregulare*. This highlights the complexity of the outcome of the interaction of different bacteria and different *Pythium* species. It should not be assumed that an agent that controls one pathogen will have the same or similar impact on other pathogens in the same genus.

Pythium disease suppression was not observed with any of the three bacterial isolates used in the greenhouse experiment. In systems inoculated by placing *Pythium* in water, including systems inoculated with bacterial isolates, there was a tendency toward enhanced *Pythium aphanidermatum*-caused disease in geranium, although this was not significant. In terms of disease severity rather than incidence, the most severe symptoms were observed in the experimental units inoculated with any of the three bacterial isolates and *Pythium* directly in the water reservoir. Synergistic relationships between *Pythium* and bacterial isolates have been observed previously suggesting that bacteria may predispose plants to *Pythium* (Paulitz et al., 1992). This suggests that the bacteria-*Pythium* interaction is complex and may not be predicted by in vitro tests (Folman et al., 2003; Pliego et al., 2011). This is an important area of research because it raises questions about the outcome of the introduction of bacteria into new environments, especially when bacteria-fungi and bacteria-*Pythium* interactions are discovered.

In conclusion, we found that a variety of heterotrophic bacteria reside in recycled irrigation in commercial greenhouses in Pennsylvania. The ecosystem sustained bacteria that inhibited, attached to, or enhanced *Pythium* hyphae in vitro, but these interactions did not occur in greenhouse plant inoculation studies. In the present research, three bacteria were selected from the microbial community found in RIW from commercial greenhouses. They were introduced into what was probably a much simpler, less microbially diverse environment in an experimental greenhouse. The in vitro effects of the individual bacteria on *Pythium* did not coincide with their effects under greenhouse conditions. Further investigations are needed to determine whether a more complex microbial community would have different effects on *Pythium* and on disease development. Investigations such as this will become more important as more greenhouse growers adopt RIW technologies.

Literature Cited

- Berger, F., H. Li, D. White, R. Frazer, and C. Leifert. 1996. Effect of pathogen inoculum, antagonist density, and plant species on biological control of Phytophthora and Pythium damping-off by *Bacillus subtilis* Cot1 in high-humidity fogging glasshouses. *Phytopathology* 86:428-433.
- Brooks, D.S., C.F. Gonzalez, D.N. Appel, and T.H. Filer. 1994. Evaluation of endophytic bacteria

- as potential biological control agents for oak wilt. *Biol. Control* 4:373–381.
- Chen, C.Q., R.R. Belanger, N. Benhamou, and T.C. Paulitz. 1998. Induced systemic resistance (ISR) by *Pseudomonas* spp. impairs pre- and post-infection development of *Pythium aphanidermatum* on cucumber roots. *Eur. J. Plant Pathol.* 104:877–886.
- Chen, M.H., A.L. Jack, I.C. McGuire, and E.B. Nelson. 2012. Seed-colonizing bacterial communities associated with the suppression of *Pythium* seedling disease in a municipal biosolids compost. *Phytopathology* 102:478–489.
- Cole, J.R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R.J. Farris, A.S. Kulam-Syed-Mohideen, D.M. McGarrell, T. Marsh, G.M. Garrity, and J.M. Tiedje. 2009. The Ribosomal Database Project: Improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* 37:141–145.
- Deniel, F., P. Rey, M. Cherif, A. Guillo, and Y. Tirilly. 2004. Indigenous bacteria with antagonistic and plant-growth-promoting activities improve slow-filtration efficiency in soilless cultivation. *Can. J. Microbiol.* 50:499–508.
- Dykstra, M.J. 1992. Biological electron microscopy: Theory, techniques, and troubleshooting. Plenum Press, New York, NY.
- Folman, L.B., J. Postma, and J.A. Van Veen. 2003. Inability to find consistent bacterial biocontrol agents of *Pythium aphanidermatum* in cucumber using screens based on ecophysiological traits. *Microb. Ecol.* 45:72–87.
- Gardener, B.B.M. 2007. Diversity and ecology of biocontrol *Pseudomonas* spp. in agricultural systems. *Phytopathology* 97:221–226.
- Ho, H.H. 1986. *Pythium dimorphum* from Rhododendron. *Mycopathologia* 93:141–145.
- Hoitink, H.J., A.G. Stone, and D.Y. Han. 1997. Suppression of plant diseases by composts. *HortScience* 32:184–187.
- Hong, C.X. and G.W. Moorman. 2005. Plant pathogens in irrigation water: Challenges and opportunities. *Crit. Rev. Plant Sci.* 24:189–208.
- Hu, J.H., C.X. Hong, E.L. Stromberg, and G.W. Moorman. 2008. Mefenoxam sensitivity and fitness analysis of *Phytophthora nicotianae* isolates from nurseries in Virginia, USA. *Plant Pathol.* 57:728–736.
- Iwamoto, T., K. Tani, K. Nakamura, Y. Suzuki, M. Kitagawa, M. Eguchi, and M. Nasu. 2000. Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. *FEMS Microbiol. Ecol.* 32:129–141.
- Kong, P., B.W.K. Lee, Z.S. Zhou, and C.X. Hong. 2010. Zoospore plant pathogens produce bacterial autoinducer-2 that affects *Vibrio harveyi* quorum sensing. *FEMS Microbiol. Lett.* 303:55–60.
- Lauro, F.M., D. McDougald, T. Thomas, T.J. Williams, S. Egan, S. Rice, M.Z. DeMaere, L. Ting, H. Ertan, J. Johnson, S. Ferreira, A. Lapidus, I. Anderson, N. Kyrpides, A.C. Munk, C. Detter, C.S. Han, M.V. Brown, F.T. Robb, S. Kjelleberg, and R. Cavicchioli. 2009. The genomic basis of trophic strategy in marine bacteria. *Proc. Natl. Acad. Sci. USA* 106:15527–15533.
- Leben, C. 1984. Spread of plant pathogenic bacteria with fungal hyphae. *Phytopathology* 74:983–986.
- Madden, L.V., G. Hughes, and F. Van Den Bosch. 2007. The study of plant disease epidemics. APS Press, St. Paul, MN.
- Martin, F.N. and J.E. Loper. 1999. Soilborne plant diseases caused by *Pythium* spp: Ecology, epidemiology, and prospects for biological control. *Crit. Rev. Plant Sci.* 18:111–181.
- Mavrodi, O.V., N. Walter, S. Elateek, C.G. Taylor, and P.A. Okubara. 2012. Suppression of Rhizoctonia and *Pythium* root rot of wheat by new strains of *Pseudomonas*. *Biol. Control* 62:93–102.
- Morita, Y. and M. Tojo. 2007. Modifications of PARP medium using fluzinam, miconazole, and nystatin for detection of *Pythium* spp. in soil. *Plant Dis.* 91:1591–1599.
- Nelson, E.B., W.L. Chao, J.M. Norton, G.T. Nash, and G.E. Harman. 1986. Attachment of *Enterobacter cloacae* to hyphae of *Pythium ultimum*, possible role in the biological control of *Pythium* preemergence damping-off. *Phytopathology* 76:327–335.
- Nguyen Duc, C., M.H. Nicolaisen, J. Sorensen, and S. Olsson. 2011. Hyphae-colonizing *Burkholderia* sp.—A new source of biological control agents against sheath blight disease (*Rhizoctonia solani* AG1-IA) in rice. *Microb. Ecol.* 62:425–434.
- Ofek, M., Y. Hadar, and D. Minz. 2012. Ecology of root colonizing *Massilia* (Oxalobacteraceae). *PLoS One* 7.
- Pane, C., A. Piccolo, R. Spaccini, G. Celano, D. Vilecco, and M. Zaccardelli. 2013. Agricultural waste-based composts exhibiting suppressivity to diseases caused by the phytopathogenic soil-borne fungi *Rhizoctonia solani* and *Sclerotinia minor*. *Appl. Soil Ecol.* 65:43–51.
- Paulitz, T.C., T. Zhou, and L. Rankin. 1992. Selection of rhizosphere bacteria for biological control of *Pythium aphanidermatum* on hydroponically grown cucumber. *Biol. Control* 2:226–237.
- Pliego, C., C. Ramos, A. De Vicente, and F.M. Cazorla. 2011. Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. *Plant Soil* 340:505–520.
- Postma, J. 2009. The status of biological control of plant diseases in soilless cultivation, p. 133–146. In: Recent developments in management of plant diseases. Springer, Torino, Italy.
- Postma, J., B.P.J. Geraats, R. Pastoor, and J.D. Van Elsland. 2005. Characterization of the microbial community involved in the suppression of *Pythium aphanidermatum* in cucumber grown on rockwool. *Phytopathology* 95:808–818.
- Postma, J., L.H. Stevens, G.L. Wiegers, E. Davelaar, and E.H. Nijhuis. 2009. Biological control of *Pythium aphanidermatum* in cucumber with a combined application of *Lysobacter* enzymes strain 3.1T8 and chitosan. *Biol. Control* 48:301–309.
- Queipo-Ortuno, M.I., J.D.D. Colmenero, M. Macias, M.J. Bravo, and P. Morata. 2008. Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis. *Clin. Vaccine Immunol.* 15:293–296.
- Scheublin, T.R., I.R. Sanders, C. Keel, and J.R. Van Der Meer. 2010. Characterisation of microbial communities colonising the hyphal surfaces of arbuscular mycorrhizal fungi. *ISME J.* 4:752–763.
- Schmidt, T.M. and A.E. Konopka. 2009. Physiological and ecological adaptations of slow-growing, heterotrophic microbes and consequences for cultivation, p. 101–120. In: Epstein SS (ed.). *Uncultivated microorganisms*. Springer, Berlin, Germany.
- Siala, A. and T.R.G. Gray. 1974. Growth of *Bacillus subtilis* and spore germination in soil observed by fluorescent antibody techniques. *J. Gen. Microbiol.* 81:191–198.
- Silva, H.S.A., J.P.L. Tozzi, C.R.F. Terrasan, and W. Bettiol. 2012. Endophytic microorganisms from coffee tissues as plant growth promoters and biocontrol agents of coffee leaf rust. *Biol. Control* 63:62–67.
- Sokal, R.R. and F.J. Rohlf. 2012. *Biometry*. W.H. Freeman and Co., New York, NY.
- Uzuhashi, S., M. Tojo, and M. Kakishima. 2010. Phylogeny of the genus *Pythium* and description of new genera. *Mycoscience* 51:337–365.
- Valerio, E., S. Chaves, and R. Tenreiro. 2010. Diversity and impact of prokaryotic toxins on aquatic environments: A review. *Toxins* 2:2359–2410.
- Van Os, G.J. and J.H. Van Ginkel. 2001. Suppression of *Pythium* root rot in bulbous Iris in relation to biomass and activity of the soil microflora. *Soil Biol. Biochem.* 33:1447–1454.
- Watanabe, H., K. Kageyama, Y. Taguchi, H. Horinouchi, and M. Hyakumachi. 2008. Bait method to detect *Pythium* species that grow at high temperatures in hydroponic solutions. *J. Gen. Plant Pathol.* 74:417–424.
- Weller, D.M., J.M. Raaijmakers, B.B.M. Gardener, and L.S. Thomashow. 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* 40:309–348.
- Wong, P.T.W. and D.M. Griffin. 1976. Bacterial movement at high matric potentials. 1. Artificial and natural soils. *Soil Biol. Biochem.* 8:215–218.