POTENTIAL BIOLOGICAL CONTROL *PSEUDOMONAS* sp. PCI2 AGAINST DAMPING-OFF OF TOMATO CAUSED BY *SCLEROTIUM ROLFSII*

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SUMMARY

Fluorescent *Pseudomonas* spp. isolated from the roots of healthy tomato plants were screened for their antagonistic activities against *Sclerotium rolfsii*, *Alternaria alternata* and *Fusarium solani*, three phytopathogenic fungi of tomato and pepper. They were tested for phosphate solubilization ability and production of siderophores, hydrolytic enzymes, indole 3-acetic acid and hydrogen cyanid. The isolates were also characterized based on biochemical (API 20NE test) and genotypic (ERIC-PCR fingerprinting) features. A *Pseudomonas* sp. strain denoted PCI2 was chosen as a potential candidate for controlling tomato damping-off caused by *Sclerotium rolfsii*. PCI2 was identified at the genus level with a 16S rDNA partial sequence analysis and its phylogenetic relationship with previously characterized *Pseudomonas* species was determined. PCI2 clustered with the *P. putida* species. Growth chamber studies resulted in statistically significant increases in plant stand (29%) as well as in root dry weight (58%). PCI2 was able to establish itself and survive in tomato rhizosphere after 40 days, following planting of bacterized seeds. PCI2 is a potential biological control agent that may contribute to the protection of tomato plants against damping-off caused by *S. rolfsii*.

Key words: fluorescent *Pseudomonas*, biological control, *Sclerotium rolfsii*, tomato plants

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is the second leading vegetable crop worldwide with a production of *ca.* $1x10^6$ tons on a surface area of $3.7x10^6$ ha (FAO, 2003). Due to increasing demand, tomato has a great potential for increased commercialization. More efficient tomato production requires better knowledge of its pathogens and control methods. Tomato plants are susceptible, among other fungi, to *Sclerotium rolfsii*, a soil-borne pathogen that attacks over 500 different plant species (Wydra, 1996). *S. rolfsii* is a major problem in tomato crops in the warm, moist tropical regions of the world (Aycock, 1966), causing damping-off in nursery seedlings as well as stem rot, wilting and blight in adult plants (Tindall, 1983; Chamswarng *et al*., 1992; Flores-Moctezuma *et al*., 2006), with consistent loss of production (De Curtis *et al*., 2010).

Synthetic chemical fungicides have long been used to reduce the incidence of plant diseases. However, they are costly, can have negative effects on the environment, and may induce pathogen resistance. Consequently, biological control, including the use of microorganisms or their antibiotics, offers an attractive alternative or supplement to pesticides for the management of plant diseases without the negative impact of chemical control (Wang *et al*., 1999). *Pseudomonas* spp. are common soil bacteria easily cultured from most agricultural soils and plant rhizospheres. They have been studied in considerable detail because of their ability to promote plant growth, either by directly stimulating the plant or by suppressing pathogens (Haas and Défago, 2005; Carlier *et al*., 2008; Rovera *et al*., 2008; Rosas *et al*., 2009; Rodriguez and Pfender, 1997; Ross *et al*., 2000; Srinivasan *et al*., 2009).

Several research groups have tested the efficacy of antagonistic microbes for the control of *S. rolfsii* (Madi *et al*., 1997; Tsahouridou and Thanassoulopoulos, 2002; Errakhi *et al*., 2007). which induces diseases difficult to control because of the production of sclerotia that represent resistant survival structures (Elad, 1995). Fluorescent pseudomonads have been reported as promising biological control agents against *S. rolfsii* in betelvine (*Piper betle* L.) (Singh, 2003) and bean (*Phaseolus vulgaris* L.) (De La Fuente *et al*., 2004), among others. Our goal was to test the application of a *Pseudomonas* isolate, isolated from tomato rhizosphere, for protecting this plant from *S. rolfsii*.

The objectives of this study were: (i) to characterize biochemically and genotypically fluorescent *Pseudomonas* isolated from tomato rhizosphere and (ii) to show the pathogenicity of *S. rolfsii* to tomato plants and

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evaluate the potential of *Pseudomonas* sp. PCI2, to control tomato damping-off caused by this fungus.

MATERIALS AND METHODS

Microorganisms and media. Fluorescent *Pseudomonas* spp. were isolated from roots of healthy tomato plants (*L. esculentum* Mill.) from the province of Córdoba, Argentina. To this end, non rhizosphere soil was removed from the root system of the plants; roots were placed into 10 ml of sterile 0.9% NaCl solution and vortexed for 10 min in order to detach the associated rhizosphere soil. Serial dilutions of the resulting root wash were plated on King´s B agar medium (KB) (King *et al*., 1954) amended with ampicillin (100 µg ml^{-1}) and cycloheximide (75 µg ml^{-1}) (Simon and Ridge, 1974). Plates were incubated at 28ºC for 24-48 h, at which time fluorescent colonies were observed under UV light (354 nm). All bacterial cultures were stored at –20 ºC in tryptic soy broth (TSB) amended with 20% glycerol. Fungal pathogens (from the fungal collection of the Laboratory of Plant-Microbe Interactions, Universidad Nacional de Río Cuarto) used were *Sclerotium rolfsii*, *Alternaria alternata* and *Fusarium solani*, all isolated from diseased tomato and pepper plants. Fungi were maintained on potato dextrose agar (PDA) at room temperature or at 4 ºC, and subcultured periodically.

Biochemical characterization. Bacterial characterization was carried out on the basis of colony morphology, Gram stain, oxidase test, production of acids from 1% glucose in OF basal medium (Hugh and Leifson, 1953), and analysis with the API 20NE biochemical test plus computer software (BioMèrieux, France). The API 20NE system facilitates the identification within 48 h of non-fastidious Gram-negative rods not belonging to the *Enterobacteriaceae.* The API 20NE strip consists of microtubes containing dehydrated media and substrates. The media microtubes containing conventional tests were inoculated with a bacterial suspension which reconstituted the media. After incubation, the metabolic end products were detected by indicator systems or the addition of reagents. The substrate microtubes contained assimilation tests and were inoculated with a minimal medium. If the isolates were capable of utilizing the corresponding substrate, they grew.

DNA Preparation and ERIC-PCR analysis. DNA was extracted according to Walsh *et al.* (1991). DNA concentration of the samples was approximately 5 ng μl^{-1}. Isolates were genotypically characterized by Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR), using primers E_1 (5'-ATGTAAGCTC-CTGGGGATTCAC-3') and E_2 (5'-AAGTAAGT-GACTGGGGTGAGCG-3'). ERIC-PCR was performed in 12 µl reaction mixture containing 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each nucleotide (Promega, USA), 0.3 µM of each primer, 1 U of *Taq* DNA polymerase (Promega, USA) and 3.6 µl of template DNA solution. The temperature profile was as follows: initial denaturation at 95°C for 1 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 65°C for 8 min, and a final extension step at 68°C for 16 min. PCR-amplifications were performed in a Mastercycler gradient block (Eppendorf, Germany). The ERIC amplification products in 6 µl sub-samples were separated by horizontal electrophoresis through 1.8% agarose gels and stained with SYBR Green.

Evaluation of isolates for biological control and growth promotion. *P-solubilization and production of siderophores and indole-3-acetic acid (IAA).* Phosphate solubilizing ability of bacterial isolates was detected according to Rosas *et al.* (2006). Production of siderophores was detected by spot inoculating the isolates on Chrome Azurol S (CAS) agar plates (Alexander and Zuberer, 1991). IAA production was analyzed in nutrient broth according to Patten and Glick (2002), with or without 200 µg ml^{-1} of L-tryptophan.

Production of hydrolytic enzymes and hydrogen cyanide (HCN). Proteolytic activity was detected by growing the isolates on a medium composed of 1% casein and 2.3% agar dissolved in Castañeda medium (Castañeda-Agulló, 1956). Plates were incubated for 48 h at 28ºC. Casein hydrolysis was detected by the formation of a whitish, opaque halo (coagulated casein) around a translucent area (totally hydrolyzed casein), surrounding the colony. Isolates were also tested for their ability to produce extracellular chitinases in a liquid medium prepared with 2% chitin from crab shells (w/v) in tap water (Rojas Avelizapa *et al.*, 1999). To determine cellulolytic activity, carboxymethyl cellulose (CMC) was incorporated at 0.1% into the YEMA–0.2% mannitol agar plates (Zorreguieta *et al.*, 1999). Production of hydrogen cyanide was assayed according to Bakker and Schipper (1987).

***In vitro* screening for antibiosis against fungal pathogens.** *Antagonism in dual culture.* Isolates were tested against *S. rolfsii*, *A. alternata* and *F. solani* in plate bioassays. *A. alternata* and *F. solani* conidia were harvested from the surface of plates by suspending in sterilized distilled water (SDW) and their concentration was determined with a Neubauer chamber (Cota *et al*., 2007). Plates containing the media to be tested (KB, PDA) were prepared. Then, an agar over-layer containing the target fungus immobilized at a concentration of 10^4-10^5 conidia ml^{-1} was placed on the medium. The methodology described by Montesinos *et al.* (1996) was followed in order to prepare the overlay, using 0.7%

agar. Four ml of the medium were placed in screw-capped test tubes that, once sterilized, were kept inside of a water bath at 40ºC. Next, 100 µl of a target conidia suspension were added to each test tube, whose content, after vortexing was homogeneously distributed on a plate containing the same culture medium. The bacterial isolates tested were sown by gently touching the agar surface with a sterile toothpick, previously inoculated by touching the surface of a single colony. Plates were incubated for 72 h at 28ºC. The degree of inhibition in each medium was measured by the halo with no fungal growth around the bacterial isolate. The average of three replicates was considered as a measure of the inhibition halo. To screen for potential antagonism against *S. rolfsii*, mature sclerotia were removed from the surface of 15-day-old cultures with sterile forceps and four were immediately placed around the edges and one in the center of a plate 24 h after the stab-inoculation of four bacterial isolates. The experiment was conducted twice.

Mycelial growth inhibition. Isolates were tested for antifungal activity on KB and PDA according to Rabindran and Vidyasekaran (1996). Briefly, isolates were streaked on 1/3 of a Petri plate containing KB or PDA. A mycelial disc (9 mm diameter) of a 8-15 day-old-culture of an actively growing target fungus was equidistantly placed on the opposite side of the Petri plate 48 h after sowing the bacterial isolate. Average inhibition zone (mm). i.e. the distance between the edge of the fungal mycelium and the isolate, was determined using three replicates for each isolate on each medium after 7 days of incubation at 28ºC. Plates with fungal pathogens on one side that were not inoculated with bacterial isolates served as controls. The experiment was repeated twice.

Genotypic identification and characterization of isolate PCI2. Amplification and sequencing of the 16S rRNA gene was performed by MACROGEN (Korea) The resulting 780 bp sequence was analyzed by means of the BLASTN algorithm (Altschul *et al.*, 1997), available in GenBank (http://www.ncbi.nlm.nih.gov/), to perform a homology study. In addition, the sequence of isolate PCI2 was aligned, using the BioEdit *V* 7.0.9.0 program, with sequences retrieved from the GenBank database. The GenBank accession Nos are AF094742 (*P. putida* ATCC 17522), AY559493 (*P. putida* UW4), FN313522 (*P. putida* ZR2-15), AJ278813 (*P. fluorescens* Q2-87), Z76673 (*P. chlororaphis* DSM 50083T), AJ278814 (*P. fluorescens* F113), AJ278812 (*P. fluorescens* CHA0), AJ704392 (*Burkholderia* sp. Hwp-12), X99297 (*Xanthomonas campestris*). The sequence obtained in this study was deposited in the GenBank under the accession No. GU004535. PAUP* *V* 4.08b (Swofford, 1999) was used to conduct an unweighted parsimony analysis on the 16S rRNA gene sequences as combined datasets for the whole taxon matrix. For the analysis, the heuristic search option was used with 100 random sequence addition replicates, with MULPARS on, and the tree bisection-reconnection (TBR) branch swapping algorithm. Phylogenetically informative indels were coded as a fifth character state. Clade stability was assessed by 100 parsimony bootstrap replications. Tree was redrawn from PAUP tree files using TREVIEW.

Quantification of indole 3-acetic acid (IAA) in culture supernatant of *Pseudomonas* sp. PCI2. Twenty milliliters of bacterial culture grown in nutrient broth (NB) were taken during the late exponential growth phase (24 h), and centrifuged at 8,000 rpm in the cold (4ºC) for 10 min. Supernatant was acidified at pH 3.0 with acetic acid solution (1% v/v), additioned to 100 ng $^2H_5$-IAA (OlChemIm, Czech Republic) deuterated internal standard, and kept at 4ºC for 1 h. The sample was partitioned three times with 20 ml ethyl acetate. After the last partition, ethyl acetate was evaporated to dryness at 36ºC. Then, the sample was resuspended in methanol prior to passing through a pre-purification Sephadex DEAE 25 column. The fraction (eluted IAA) containing the deuterated internal standard was evaporated to dryness and resuspended in a vial containing 100 µl methanol. The vial was introduced into the autosampler of an Alliance 2695 liquid chromatographer (Waters Inc, USA) and 10 µl were injected. Chromatographic conditions were constant, with acetonitrile:water (65:35) at a flow rate of 0.2 ml min^{-1}, and the sample was further analyzed using a Quattro Ultima™ Mass Spectrometer (Micromass, UK). IAA identification was carried out by comparing the retention time of the sample with the pure standard, and quantification was performed using the MRM (Multiple Reaction Monitoring) function, following the 174/179 molecular masses and 130/135 transition masses, which correspond to endogenous/deuterated, respectively. For quantification values were obtained from the calibration curve, previously designed using IAA pure standard (Sigma, USA).

Effect of *Pseudomonas* sp. PCI2 on tomato plant growth. Preliminary assays were carried out to evaluate the response of tomato plants to PCI2. Seeds of tomato cv. Platense Italiano (Asociación Cooperativa INTA La Consulta, Argentina) were surface-disinfected for 10 min in 5% sodium hypochlorite solution (60 g l^{-1} of active chlorine), washed 10 times in sterilized distilled water (SDW), and air dried (Tsahouridou and Thanassoulopoulos, 2002). Then, 10 g of seeds were soaked for 30 min in 2.5 ml of a 10^9 CFU ml^{-1} aqueous cell suspension of *Pseudomonas* sp. PCI2. The bacterium was grown under shaking (80 rpm) in KB broth for 48 h at 28ºC (Jayaraj *et al.*, 2007). Plastic pots (15 cm diameter; 25 cm height) were filled with a mixture of soil:sand:perlite (at 2:1:1 w/w/w), previously sterilized

by heating at 180ºC for 2 h on four consecutive days. Then, seven inoculated seeds were sown into the soil mix in each pot. Pots containing non-bacterized seeds were used as control. Pots were incubated in a growth chamber under controlled conditions: 16 h light at 28±2ºC, 8 h dark at 16± 2ºC (light intensity of 220 ºE m^{-2} s^{-1}), and watered once daily with distilled water (20-100 ml per pot). Shoot and root dry weights (72 h at 70ºC) were determined 40 days after seedling emergence. Pots were arranged in a completely randomized design with six replicates per treatment and the experiment was repeated twice. In addition, the survival of *Pseudomonas* sp. PCI2 in the rhizosphere of tomato plants was determined according to a modification of the procedure described by Landa *et al*. (2004) at 1h, and 10, 25 and 40 days after sowing. Briefly, a seedling from each treatment was carefully removed and roots were gently shaken to remove all but tightly adhering potting mixture; 1 g of the adhering rhizosphere mixture was collected and placed into 9 ml of sterile 0.9% NaCl solution. Serial dilutions of the suspension were vortexed and plated onto KB agar amended with ampicillin and cycloheximide (Simon and Ridge, 1974); plates were incubated for 48 h at 28ºC. The developed colonies from both treatments were counted and the number of CFU g^{-1} of mixture was calculated.

***S. rolfsii* pathogenicity and *in vivo* antagonistic activity of *Pseudomonas* sp. PCI2 in tomato plants.** Cultures of *S. rolfsii* were maintained on PDA, on which brown sclerotia formed within 8 to 10 days. Pathogen inoculum added to sterile mixture consisted of 30-day-old sclerotia which were dislodged from the surface of plates and used immediately (Papavizas and Lewis, 1989). Plastic pots (15 cm diameter; 25 cm height) were filled with 600 g of sterile mixture (soil:sand:perlite at 2:1:1 w/w/w). Each pot was then moistened with SDW and infested in the mixture surface with 30 mg of sclerotia. Pots were kept for 8 days in a growth chamber under controlled conditions (as described above). After incubation, tomato seeds were bacterized as described above and sown into the infested pots at eight seeds per pot. The four treatments were: (i) non-infested, non-bacterized healthy control (treated with SDW); (ii) infested with *S. rolfsi,* non-bacterized control; (iii) infested with *S. rolfsii* and bacterized with PCI2, and (iv) bacterized with PCI2 alone. Pots were incubated in a growth chamber under the conditions described above. Damping-off was determined by counting the total healthy stand after 40 days, compared to non-infested control plants. Shoot and root dry weights (72 h at 70ºC) were recorded from twenty randomly selected plants from each treatment. Survival of *Pseudomonas* sp. PCI2 in the rhizosphere of tomato plants was determined as described above from treatments 1 and 4. Pots were arranged in a completely randomized design. The experiment was performed twice, each with six replicates per treatment.

Statistical analyses. Results regarding the assays of effect of PCI2 on tomato plants growth were compared by means of the *t* test, while results obtained in the experiments of *in vivo* antagonistic activity of PCI2 were analyzed by using analysis of variance (ANOVA). When ANOVA showed treatment effect, the least sgnificant difference (LSD) test was applied to compare means at $P<0.05$. All data were subjected to statistical analysis using Statgraphics plus software for Windows *V 4.1* (Statistical Graphics, USA).

RESULTS

Characterization of bacterial isolates. All isolates from tomato roots were Gram-negative rods, oxidase-positive and capable of metabolizing glucose in an oxidative form. API 20NE analyses revealed that nine of them belonged to the genus *Pseudomonas* and one to *Ralstonia*. Results obtained with the API 20NE biochemical test for *Pseudomonas* sp. TEI1 and *Pseudomonas* sp. TR1 revealed that both isolates belong to *P. fluorescens*; moreover, these isolates had similar band profiles in the ERIC-PCR analysis. However, other isolates that were biochemically characterized as *Pseudomonas fluorescens* (P8), *P. aeruginosa* (P4) or *P. putida* (P1, P12, TBR2, PCI2 and PBR3) by means of the API 20NE test did not show similarities in their band profiles. ERIC-PCR generates multiple distinct amplification products and allows discrimination at genus, species, and isolate levels based on the electrophoretic pattern of amplification products (de Bruijn, 1992). Thus, isolates belonging to the same species were differentiated using ERIC-PCR fingerprinting.

***In vitro* screening for antibiosis against fungal pathogens.** The bacterial antagonistic effect on fungal germination depended both on target pathogen and culture medium. The strongest *in vitro* antagonism against all tested fungi was observed on KB agar. Likewise, the *in vitro* inhibition of mycelial growth also varied with culture medium and target pathogen. As observed in the germination inhibition assay, inhibition of mycelia growth of all tested fungi was more effective on KB agar. The greatest average value against *A. alternata* was 28 mm, and the lowest 21 mm. Against *F. solani*, the lowest diameter of inhibition zone, 6 mm, was obtained with isolate TR1, and the greatest, 10 mm, with isolate P6. When recording inhibition zones against *S. rolfsii*, it was observed that isolates PBR3 and TBR2 produced the lowest diameters, 2 mm, while isolate PCI2 produced the highest, 27 mm. Fungal pathogens were weakly inhibited on PDA (Table 1).

Table 1. Characteristics of *Pseudomonas* spp. and *Ralstonia* sp. isolated from healthy tomato plants.

Bacterial isolate	Diameter of inhibition zone (mm)[a]						Siderophore production[b]	Phosphate solubilization[c]	IAA (µg ml^{-1})[d]		Protease[e]	Chitinase (µM ml^{-1} NAG)
	KB			PDA								
	S.r	*A. a.*	*F. s.*	*S. r.*	*A. a.*	*F. s.*			T	NT		
P1	7bc	21ns	8abcd	6b	13a	3b	+	++	16.5	2.3	-	-
P8	7bc	28ns	7bcde	5b	9ab	4b	++	+++	14.4	2.2	1	-
TBR2	2c	24ns	7bcde	2bc	6b	6a	+	++++	20.5	2.7	-	-
TEI1	9bc	26ns	8abcd	4b	5ab	4b	++	-	16.6	2.2	-	-
TR1	16b	28ns	6de	4b	8ab	6a	++	++	17.4	2.6	-	-
P4	20b	26ns	9abc	6b	10ab	6a	+	+++	13.4	2.1	2	-
P6	15b	21ns	10a	2bc	6b	4b	++	+	6.8	0.0	1	-
P12	3c	22ns	9abc	2bc	10ab	7a	+	+++	20.4	3.0	-	-
PBR3	2c	21ns	6de	5b	5bc	4b	+	+++	19.2	2.2	-	-
PCI2	27a	27ns	7de	9a	10ab	6a	++	++++	21.5	3.5	-	2.4

[a]*S. r.*: *S. rolfsii*; *A. a.*: *A. alternata; F. s.*: *F. solani*;. Values with the same letter within the same column are not significantly different according to the LSD (P<0.05) test; ns: not significantly different.
[b]Siderophore production was compared among the bacterial isolates by measuring the diameter of orange halo produced on CAS agar plates: + = halo diameter of 1-3 mm, ++ = 3-5 mm.
[c]Diameter of clear zone formed around the bacterial colony as a result of solubilization of tri-calcium phosphate: + = 1-3 mm clear zone, ++ = 3-5 mm clear zone, +++ = 5-8 mm clear zone, ++++ = 8-10 mm clear zone.
[d]T: grown in medium with tryptophan (200 µg ml^{-1}); NT: grown in medium lacking tryptophan.
[e]According to Rojas Avelizapa *et al.* (1999): 0, colony without halo; 1, an opaque halo of 2±5 mm in diameter; 2, a halo of 5±9 mm, opaque in the periphery and somewhat translucent in the central area.

Evaluation of isolates for biological control and growth promotion. All isolates were able to respond to iron limitation producing siderophores as evidenced by the formation of yellow/orange halos around the colonies grown on CAS-agar plates. Except for TEI1, all isolates solubilized the insoluble form of phosphorus. In addition, all ten isolates produced IAA. The lowest amounts, 6.8 and 0.0 µg ml^{-1}, were produced by *Ralstonia* sp. P6 and the greatest amounts, 21.5 and 3.5 µg ml^{-1}, were produced by *Pseudomonas* sp. PCI2, with and without addition of 200 µg ml^{-1} of tryptophan to culture medium, respectively. *Pseudomonas* sp. P4, *Ralstonia* sp. P6 and *Pseudomonas* sp. P8 showed protease activity, while isolate PCI2 produced chitinase. None of the isolates showed cellulolytic activity or produced hydrogen cyanide (Table 1). *Pseudomonas* sp. PCI2 was chosen for further studies based on *in vitro* antagonistic effect against *S. rolfsii* and traits that may be involved in plant-growth promotion. IAA production by isolate PCI2 was also analyzed using a Quattro Ultima^{TM} Mass Spectrometer; under the conditions tested, *Pseudomonas* sp. PCI2 produced 4.71 µg ml^{-1} IAA after 24 h of growth.

Genotypic identification and characterization of isolate PCI2. When a 780 bp fragment of the 16S rRNA gene from isolate PCI2 was sequenced, PCI2 showed a 100% identity with sequences from species of *Pseudomonas*. The sequence of PCI2 was aligned, using PAUP, with sequences of some *Pseudomonas* spp. retrieved from GenBank (*P. putida* ATCC 17522; *P. putida* UW4; *P. putida* ZR2-15; *P. fluorescens* Q2-87; *P. chlororaphis* DSM 50083T; *P. fluorescens* F113; *P. fluorescens* CHA0). Fig. 1 shows that PCI2 clustered with *P. putida.*

Effect of *Pseudomonas* sp. PCI2 on tomato plants growth. Plants emerged from seeds inoculated with PC12 showed an increase in root dry weight (157±7.9 mg) compared to control plants (110±8.2 mg). Shoot biomass was slightly greater in inoculated plants (225±8.1 mg) than in control plants (204±7.8 mg). Furthermore, it appeared that PCI2 was able to establish and survive in the rhizosphere of tomato plants. *Pseudomonas* with similar colony morphologies to PC12 were recovered from roots of bacterized seeds at a population density of 10^6-10^7 CFU g^{-1} mixture 40 days after planting of bacterized seeds, while colony counts performed from non-bacterized control plants revealed absence of colonies morphologically similar to PCI2.

***S. rolfsii* pathogenicity and *in vivo* antagonistic activity of *Pseudomonas* sp. PCI2 in tomato plants.** In *S. rolfsii*-infested soil mix, inoculating tomato seeds with *Pseudomonas* sp. PCI2 improved seedling stand by 29% and increased shoot and root dry weight of plants over the untreated pathogen controls by 84.7 mg and 59.9 mg, respectively (Table 2). No evident differences between bacterized and control seeds were observed in non-infested potting mixture when recording plant stand; however, inoculation of seeds with PCI2 increased (P<0.05) root dry weight by 71.8 mg. Although PCI2 inoculation increased shoot dry weight by 33.6 mg, when compared to healthy controls, differences were not significant (Table 2). Fluorescent *Pseudomonas*

Table 2. Percent stand, and dry shoot and root weights following treatment of tomato plants with *S. rolfsii* and *Pseudomonas* PCI2.

Treatment[a]	Final Stand (%)[b]	Root dry weight (mg)[b]	Shoot dry weight (mg)[b]
Non-infested, non-bacterized healthy control	85a	123.2 ± 17b	207.3 ± 11b
Infested with *S. rolfsi,* non-bacterized control	41c	86.7 ± 15.2c	133.5 ± 14.8c
Infested with *S. rolfsii* and bacterized with PCI2	70b	146.6 ± 10.1b	218.2 ± 18.3b
Bacterized with PCI2 alone	80a	195 ± 11a	240.9 ± 12.8ab

[a]Plastic pots were filled with sterile mixture (soil:sand:perlite at 2:1:1 w/w/w). Each pot was then moistened with sterile distilled water and infested in the mixture surface with 30 mg of sclerotia. Pots were kept for 8 days in a growth chamber under controlled conditions. After incubation, tomato seeds were bacterized and sown into the infested pots at eight seeds per pot. Damping-off was determined by counting the total healthy stand after 40 days. Shoot and root dry weights were recorded from twenty randomly selected plants from each treatment.
[b]Data represent the average of two experiments ± standard deviation. Values in each column with different letters are significantly different according to the LSD test ($P<0.05$).

morphologically similar to PCI2 reached a population density of 10^7-10^8 and 10^6-10^7 CFU g^{-1} mixture after ten and forty days of experimentation, respectively, in treatment bacterized with PCI2 alone. Colony counts performed from non-infested, non-bacterized control plants revealed absence of colonies morphologically similar to PCI2.

DISCUSSION

A promising strategy for the replacement and/or supplement of chemicals is the implementation of biocontrol technology, used individually or as an integrated control component (Akrami *et al*., 2009). We investigated some physiological traits related to biological control and plant growth promoting activity to determine the potential of bacterial isolates from tomato root system as biocontrol and/or biofertilizing agents, chosing an isolate that may have great potential for controlling *S. rolfsii* in tomato plants. Previous research had indicated that methods employed to isolate rhizobacteria play an important role in the identification of potential biological control agents, and that the isolates should be obtained from the rhizosphere of the target crop (Williams and Asher, 1996).

Plant growth-promoting properties of ten isolates were assessed by determining their ability to produce IAA, siderophores, hydrolytic enzymes and HCN, to solubilize tri-calcium phosphate, and to inhibit fungal pathogens on KB and PDA media. The antagonistic properties of the isolates were influenced by culture medium composition and the fungal pathogen. Results of *in vitro* inhibition of fungal germination and reduction of mycelial growth were consistent with those of Borowicz and Saad Omer (2000), who proposed that differences between media could result in alterations in metabolites produced, or their relative concentrations. On the whole, all isolates showed inhibitory activity against the tested pathogenic fungi. In particular, *Pseudomonas* sp. PCI2 reduced mycelial growth of *S. rolfsii* up to 40%.

All isolates were able to mobilize iron from chelating agents added to CAS agar medium, and most of them expressed their maximum inhibitory activity on KB in dual culture antagonism assays. Moreover, antagonistic activity of the isolates strongly declined as iron concentration in KB increased (data not shown). Thus, involvement of siderophores in their inhibitory activities is suggested. Chitinase production was only detected in *Pseudomonas* sp. PCI2; nevertheless, PCI2 did not show cyanogenic activity.

Walsh *et al*. (2001) emphasized the need to investigate *in situ* colonization in the rhizosphere to determine the potential of a *Pseudomonas* isolate as an effective biological control agent (BCA). Moreover, Botelho and Hagler (2006) stated that the use of fluorescent Pseudomonads as plant growth-promoting rhizobacteria (PGPR) and/or BCAs requires a thorough understanding of the plant response to the presence of these introduced bacteria at high concentrations (as in the inoculants). Forty days after sowing inoculated seeds, PCI2 reached a population density of 10^6-10^7 CFU g^{-1} soil under growth chamber conditions. In this system, PCI2 did not appear to negatively affect the development of tomato plants, but it enhanced growth of the root system by over 50%.

P solubilization and IAA production are often associated with plant growth promotion (Richardson, 2001). Previous reports have indicated that IAA synthesis is related to plant growth stimulation by microorganisms, including *P. putida* (Patten and Glick, 2002). IAA is the most common natural auxin found in plants and its positive effect on root growth and morphology is believed to increase the access to more nutrients in the soil (Vessey, 2003). PCI2 solubilized P under the conditions tested and was able to synthesize IAA, which supports the hypothesis that microbial IAA and available P may

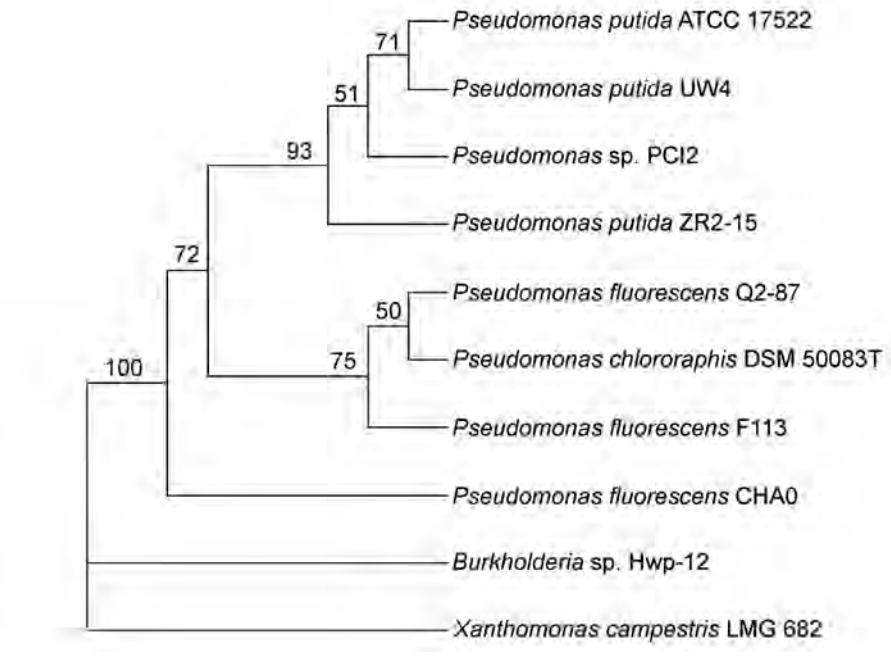

Fig. 1. Phylogenetic tree of *Pseudomonas* sp. PCI2 and related *Pseudomonas* species based on 16S rDNA sequences. The phylogenetic tree was constructed based on the percent difference in the genetic relationships between related strains from GenBank. PAUP* *V* 4.08b was used to conduct the unweighted parsimony analysis on the 16S rRNA gene sequences as combined datasets for the whole taxon matrix. The heuristic search option was used with 100 random sequence addition replicates, with MULPARS on, and the tree bisection-reconnection (TBR) branch swapping algorithm. Phylogenetically informative indels were coded as a fifth character state. Clade stability was assessed by 100 parsimony bootstrap replications.

be involved in the growth stimulation observed in our growth chamber assays.

Several reports have shown the potential of *Pseudomonas* species as BCAs for controlling plant and fruit diseases (Walsh *et al*., 2001; Okubara *et al*., 2004; Botelho and Hagler, 2006; Jayaraj *et al*., 2007; Trivedi *et al*., 2008). *Pseudomonas* sp. PCI2 showed *in vitro* inhibition of three fungal phytopathogens. In addition, PCI2 showed promise to control tomato damping-off caused by *S. rolfsii* by increasing plant stand in growth chamber assays by 29%. Thus, the controlling effect of PCI2 was reflected in higher values of shoot (63%) and root (almost 70%) dry weights, observed in the infested-bacterized treatment, when compared to pathogen controls.

Production of siderophores and chitinases are two factors that may be involved PC12 biological control activity. Indeed, it is known that chitinolytic activity and siderophore production are correlated with antifungal activity (Castoria *et al*., 2001; Kamensky *et al*., 2003; Quecine *et al*., 2008). In addition, PCI2 is capable of solubilizing phosphate and producing IAA, characteristics that may enhance its potential use as an effective biological control agent to contribute to the control of tomato damping-off caused by *S. rolfsii*. Further work is underway in order to elucidate the specific factors involved in both growth stimulation and protection of tomato plants by *Pseudomonas* sp. PCI2.

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