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Selection of a new *Pseudomonas chlororaphis* strain for the biological control of *Fusarium oxysporum* f. sp. *radicis-lycopersici*

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Summary. Fluorescent pseudomonads possess several physiological characteristics exploitable for the biological control of phytopathogenic fungi. A group of 11 pseudomonads able to inhibit tomato pathogenic fungi *in vitro* were identified using the Biolog test and the phylogenetic analysis of *recA*. Strain M71 of *Pseudomonas chlororaphis* was selected as a new potential biocontrol agent. This strain drastically reduced *Fusarium oxysporum* f. sp. *radicis-lycopersici* pathogenicity on tomato plantlets in seed assays and greenhouse trials. Moreover, the strain produced several important secondary metabolites, including proteases, siderophores and antibiotics. The presence of a region involved in phenazine production and the biosynthesis of *N*-acyl homoserine lactones were also assessed.

Key words: *recA*, phenazines, quorum sensing,

Introduction

Biological control of phytopathogenic fungi is an attractive alternative to conventional chemical control approaches, and may be accomplished through the selection and exploitation of bacterial strains antagonistic to specific plant pathogens. The genus *Pseudomonas* contains a number of strains that share traits useful for plant protection (Haas and Defago, 2005) such as the ability to persist on the roots of cultivated plants and to produce antibiotics and siderophores. Antibiotics belonging to the family of phenazines (PHZ) and phloroglucinols (PGL) (Thomashow *et al*., 1990; Raaijmakers and Weller, 1998) synthesized by fluorescent pseudomonads were shown to be the primary mechanism responsible for the natural suppression of wheat take-all (Haas and Defago, 2005; Weller, 2007). Several studies have shown the broad host activities of these compounds, revealing their effectiveness towards other phytopathogenic fungi such as *Thielaviopsis basicola, Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Rhizoctonia solani* (Stutz and Defago, 1986; Duffy and Defago, 1997; Chin-A-Woeng *et al*., 1998; Shanmugaiah *et al*., 2010).

Production of PHZ and PGL is positively controlled by a social communication mechanism termed *quorum sensing* (Fuqua *et al*., 1994), which depends on bacterial cell density perceived through the production of small diffusible signals. Particularly, PHZ production in pseudomonads relies on

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the synthesis of *N*-acyl homoserine lactones (AHL) mediated by synthase enzymes belonging to the LuxI family (Wood and Pierson, 1996; Chin-A-Woeng *et al*., 2001). Once these signals reach a threshold concentration within cells, they bind to transcriptional regulators of the LuxR family, which in turn bind to the PHZ promoter region activating transcription of the PHZ biosynthetic operon (Pierson *et al*., 1994; Chin-A-Woeng *et al*., 2001).

The present study deals with the selection of new potential biological control agents among a group of fluorescent pseudomonads isolated from tomato rhizosphere. Bacterial strains able to inhibit *F. oxysporum* f. sp. *lycopersici* (*Fol*) and *F. oxysporum* f. sp. *radicis-lycopersici* (*Forl*) were identified through a combination of phylogenetic analysis of the *recA* gene and Biolog test. We further characterized these fluorescent pseudomonads in regards to known traits associated with biological control, including PHZ, 2,4-diacetylphloroglucinol (2,4-DAPG) and diffusible AHL production. The selection of a new *P. chlororaphis* strain able to effectively control *F. oxysporum* f. sp. *radicis-lycopersici in vivo* represented the main outcome of this work.

Materials and methods

Bacterial isolation from tomato rhizosphere

Twenty tomato plants from open fields and greenhouses of different farms located in five horticultural districts of Campania (Southern Italy), were processed for the isolation of fluorescent pseudomonads.

Five grams of roots per plant were cut, put in 45 mL of sterile distilled water and incubated under agitation at room temperature for 1 h. The suspension was serially diluted and 100 µL aliquots from dilutions 10^{-3}, 10^{-4} and 10^{-5} were respectively spread onto three King's B Agar plates amended with 100 mg L^{-1} cycloheximide. After incubation at 27°C for 48 h, ten bacterial colonies that appeared similar to known fluorescent pseudomonads were selected from each root sample, purified onto LB agar plates and stored in glycerol (20%) at -20°C. The selected strains were evaluated *in vitro* in dual cultures against *Fol* and *Forl* from the collection of the Dipartimento di Arboricoltura, Botanica e Patologia Vegetale. Briefly, a volume of 50 µL of overnight cultures was spotted at two opposite sides of a potato dextrose agar (PDA) plate coinoculated at the centre with a plug of 5 mm of mycelium cut from the edge of a young colony of *Fol* or *Forl* grown on PDA medium. PDA plates inoculated with mycelium plugs only were used as experimental controls. Inhibition of mycelial growth was evaluated after 7 days at 24°C. Each test was performed in triplicate.

Identification procedure

The isolates able to reduce *Fol* and *Forl* mycelia growth were identified using the Biolog Identification System and phylogenetic analysis of *recA*. The Biolog Identification System (Biolog, Hayward, CA, USA) is based on the analysis of the utilization of 95 carbon sources by the bacterial strains. Biolog GN2 plates were prepared according to the manufacturer's instructions and were scored after 24 hours at 27°C in order to obtain the physiological profile of each strain. This test was repeated at least twice for each isolate.

Phylogenetic analysis of nucleotide sequences coding for *recA* is a suitable method for identification of bacteria (Hilario *et al*., 2004). A sequence of 600 bp specific for *recA* in each isolate was amplified by using the primers pair, PCR mixture and thermal cycles described by Hilario *et al*. (2004). PCR products were sequenced by Primm s.r.l. (Milano, Italy) by using the primer set involved in PCR reactions. A BLAST search identified a collection of known *recA* sequences from fluorescent pseudomonads in GenBank. The sequences were aligned using the ClustalX programme (Thompson *et al*., 1997), and the alignment profile was then used to establish the evolutionary distances. This calculation was performed using the Kimura two parameters model (Kimura, 1983) implemented in the MEGA3 program (Kumar *et al*., 2004). The same program was used to construct the best phylogenetic tree by the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis with 3000 replicates was performed in order to assess confidence levels for the branches (Felsenstein, 1985).

Analyses of the antagonistic properties

Analysis of chitinase and siderophore production were performed according to Puopolo *et al*. (2010). Biosynthesis of secondary metabolites with antibiotic activity was assessed by growing bacte-

rial strains spotted on cellulose pellicles placed on PDA plates for 48 h at 28°C. PDA plates covered with cellulose pellicles only and pellicles inoculated with *Escherichia coli* strain DH5α were used as negative controls. Further pellicles inoculated with the antibiotic producing strains *P. chlororaphis* 30-84 and *P. fluorescens* CHA0 represented positive controls. Cellulose pellicles were removed carefully from control and pre-treated plates so as to not contaminate the underlying substrates. Plugs of 5 mm of a 24 h old *Rhizoctonia solani* culture were used to inoculate both the control and the pre-treated plates. The diameters of fungal mycelia were scored after 72 h at 24°C. Three plates were used for each strain and the tests were replicated three times.

Primer pairs Pca2a/Pca3b and Phl2a/Phl2b, respectively, were used to amplify two regions belonging to the loci involved in the biosynthesis of PHZ and 2,4-DAPG according to the procedure described by Raaijmakers *et al*. (1997). DNA extracted from bacterial strains *P. chlororaphis* 30-84 and *P. fluorescens* CHA0 were used as the positive PHZ and PGL controls, respectively, while DNA from *E. coli strain* DH5α was used as the negative control in all PCR reactions. Products of the PCR reactions were verified by agarose gel electrophoresis, purified and sequenced by Primm s.r.l (Milano, Italy) using the respective primer set utilized in the PCR reactions. Nucleotide sequences were submitted for BLAST search in order to find homologies with DNA sequences already deposited in GenBank.

Production of signal molecules

The detection of AHL quorum sensing signals was carried out using the bacterial biosensors *Agrobacterium tumefaciens* NT1 (pZLR4) and *Chromobacterium violaceum* CV026 (McClean *et al*., 1997; Cha *et al*., 1998). The latter bacterial biosensor was used in the plate T streak assay (Steindler and Venturi, 2007) while *A. tumefaciens* NT1 (pZLR4) was used in the Petri dish assay according to the procedure of Cha *et al*. (1998). Bacterial strains were scored as AHL producers if they restored violacein production in strain CV026 or promoted *lacZ* transcription in strain NT1 (pZLR4). *P. chlororaphis* strain 30-84 and *E. coli* strain DH5α were used as positive and negative controls, respectively.

Biocontrol assays

Pseudomonas chlororaphis strain M71 was evaluated for the control of *Forl* isolate F55 NA on tomato plantlets in seed assays and greenhouse trials according to published procedures (Puopolo *et al*., 2010). Briefly, the seed assays were carried out in glass tubes filled with 5 g of sterile perlite and 18 mL of sterile Hoagland solution. The following four treatments were compared: (1) untreated plants; (2) plants + M71; (3) plants + M71 + *Forl* F55 NA; and (4) plants + *Forl* F55 NA. Each treatment was composed of 20 tubes, each containing three disinfected tomato seeds (cv. Marmande). In treatments 2 and 3, seeds were dipped for 30 min in 1 mL of a 10^7 cfu mL^{-1} suspension of M71 before transferring into sterile tubes. A second bacterization with 2 mL of a 10^8 cfu mL^{-1} suspension of M71 for each tube belonging to treatments 2 and 3 was carried out 2 days later. Two days after the second bacterization, tubes in treatments 3 and 4 were inoculated with 2 mL of a 10^4 conidia mL^{-1} suspension of *Forl* isolate F55 NA.

In the greenhouse trials, 5 mL of *Forl* isolate F55 NA conidial suspension (4×10^3 conidia mL^{-1}) were mixed thoroughly with 20 g of sandy soil and placed in a single pot. Tomato seeds were coated with bacteria by dipping them in a mixture of 1% (wt/vol) methylcellulose (Sigma, USA) containing 1×10^9 cells mL^{-1} of M71. Coated seeds were dried overnight in a laminar flow cabinet. Dilution plating showed that each seed was covered by approximately 1×10^4 living cells of strain M71. Two days after *Forl* isolate F55 NA soil inoculation, one seed per pot was sown at about 1 cm depth. The same treatments described for the seed assay were adopted but, in this case, each treatment was composed of three groups of 30 plants. Seedlings were grown in a greenhouse at 21°C, 70% relative humidity, and 16 h of daylight.

Both tests were repeated at least twice. The percentage of dead plantlets due to *Forl* attacks was scored 3 weeks after pathogen inoculation and the results were analyzed using ANOVA, and the significance of differences between treatment means was compared by Tukey's test. At the end of the trials, the population levels of strain M71 onto plantlet roots were evaluated by dilution plating on LB agar plates amended with cycloheximide 100 mg L^{-1}.

Results

A group of 90 bacterial isolates sharing colony morphology similar to fluorescent pseudomonads was selected within a collection of 200 isolates obtained from tomato rhizosphere, and was screened for the ability to inhibit the mycelial growth of *Fol* and *Forl in vitro*. Eleven fluorescent pseudomonads drastically reduced the mycelial growth of both phytopathogenic fungi.

The identification approach adopted in this work allowed us to identify all 11 strains to the species level (Table 1). Analysis of the PCR-amplified portion of the *recA* genes from each of the 11 strains is shown in Figure 1. The dendrogram derived from the phylogenetic analysis of the *recA* nucleotide sequences divided the isolates into five clusters. Cluster I included all *P. fluorescens* biotypes, *P. putida* biotype B and the *P. tolaasii* species, cluster II was originated by *P. aurantiaca*, *P. aureofaciens* and *P. chlororaphis*. *Pseudomonas syringae* and *P. viridiflava* were co-located in cluster III, while cluster IV contained *P. putida* biotype A. *P. aeruginosa* was included in cluster V. Cluster I could be further divided into two subclusters named IA (regrouping *P. fluorescens* biotype A, B, C, G and *P. tolaasii*) and IB (regrouping *P. fluorescens* biotype F and *P. putida* biotype B). The 11 strains able to inhibit *Fol* and *Forl* growth were scattered over clusters I, II, IV and V.

The metabolic profiles identified through the Biolog analysis allowed us to verify the classification of the 11 strains as *Pseudomonas* species. We found substantial agreement between data achieved with this physiological test and the molecular data derived from the *recA* analysis. For instance, strains M71 and M73, identified as *P. chlororaphis* by the Biolog system, were included in cluster II of the *recA* phylogenetic tree (Table 1).

Characterization of potential biocontrol agents

Once identified, pseudomonad strains were evaluated for several properties associated with biological control. All 11 strains were able to produce siderophores, while none of them could degrade chitin (Table 2). Bacterial strains belonging to *P. aeruginosa* and *P. chlororaphis* produced siderophores, proteases and were able to totally inhibit *R. solani* growth when compared in tests to assess antibiotic production, as much as did the antibiotic producing strains *P. chlororaphis* 30-84 and *P. fluorescens* CHA0.

The detection through PCR of nucleotide sequences involved in the biosynthesis of PHZ and 2,4-DAPG was chosen as an indicator of the production of these antibiotics.

The 1,000 bp amplicon associated with PHZ production was amplified in strains belonging to *P. aeruginosa* and *P. chlororaphis*. DNA sequence analysis of the PCR product amplified from strain

Table 1. Identification of *Pseudomonas* isolates through molecular and physiological tests.

Bacterial isolates	Bacteria identification	
	recA phylogenesis	Biolog test
Af1	Cluster IA	*P. fluorescens* biotype A
M51	Cluster IA	*P. tolaasii*
M54	Cluster IA	*P. tolaasii*
M62	Cluster V	*P. aeruginosa*
M71	Cluster II	*P. chlororaphis*
M72	Cluster V	*P. aeruginosa*
M73	Cluster II	*P. chlororaphis*
M80	Cluster IB	*P. fluorescens* biotype F
M88	Cluster IV	*P. fulva*
M114	Cluster IA	*P. synxantha*
M121	Cluster IA	*P. fluorescens* biotype A

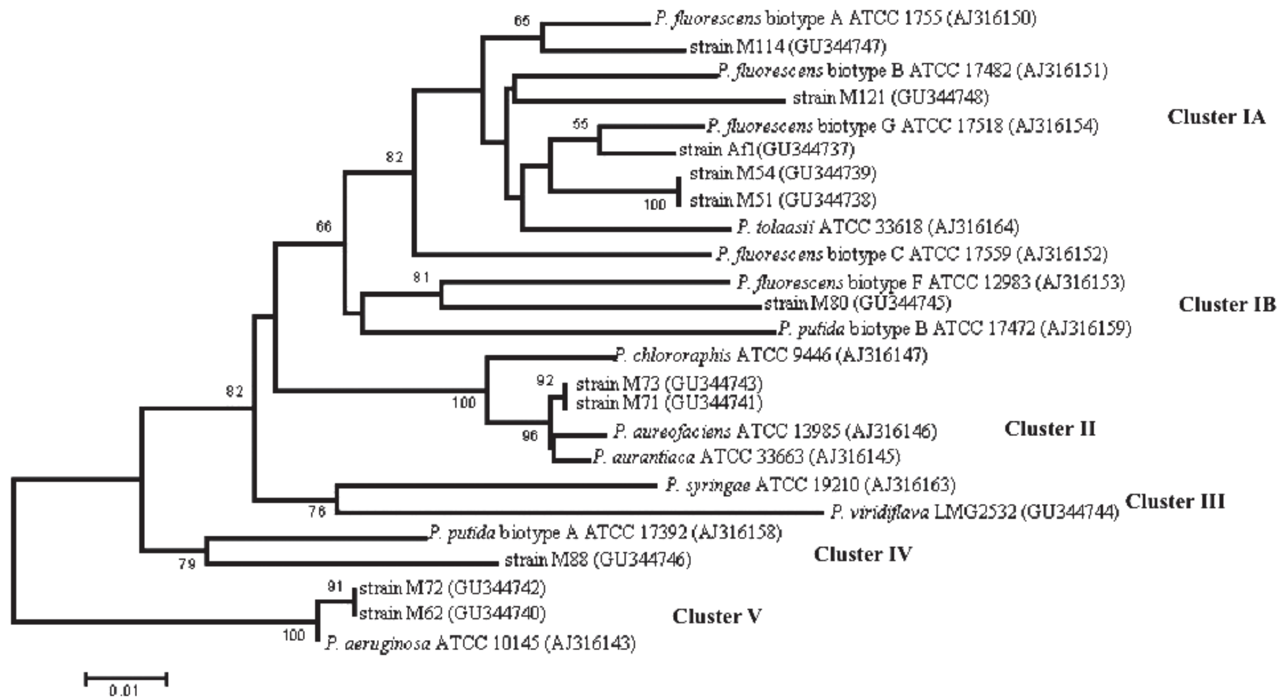

Figure 1. Phylogenetic tree derived by the analysis of *recA* gene sequences of 11 fluorescent pseudomonads using the neighbor-joining method. Evolutionary distances were calculated by using the Kimura two parameters model. Bootstrap values higher than 50 are shown at the branch points.

Table 2. Evaluation of potential mechanisms of biological control of the 11 *Pseudomonas* strains.

Bacterial strain	Siderophore production	Proteolytic activity	Chitinolytic activity	*Rhizoctonia solani* growth inhibition	2-4 DAPG	PHZ	AHL
E. coli DH5a	-[b]	-	-	-	-	-	-
P. fluorescens CHA0	+[b]	+	+	+	+	-	-
P. chlororaphis 30-84	+	+	-	+	-	+	+
P. fluorescens Af1	+	-	-	-	-	-	-
P. tolaasii M51	+	+	-	-	-	-	-
P. tolaasii M54	+	+	-	-	-	-	-
P. aeruginosa M62	+	+	-	+	-	+	+
P. chlororaphis M71	+	+	-	+	-	+[a]	+
P. aeruginosa M72	+	+	-	+	-	+	+
P. chlororaphis M73	+	+	-	+	-	+	+
P. fluorescens M80	+	+	-	-	-	-	-
P. fulva M88	+	-	-	-	-	-	-
P. synxantha M114	+	-	-	-	-	-	-
P. fluorescens M121	+	+	-	-	-	-	-

[a] Accession number of partial sequence of phenazine operon of strain M71 (GU344736).
[b] -, absence of activity; +, presence of activity.

M71 showed it was 93% identically to the region *phzF-phzA* of *P. chlororaphis* strain 30-84 (Table 2). The PCR primers specific for 2,4-DAPG production failed to amplify any sequence in the 11 strains, while specific amplicon for 2,4-DAPG was detected in the case of *P. fluorescens* strain CHA0 used as positive control.

Production of AHL signals was assessed using the bioreporter strains *A. tumefaciens* NT1 (pZLR4) and *C. violaceum* CV026. Only *P. aeruginosa* and *P. chlororaphis* strains were able to produce a range of signal molecules recognized by these bioreporters as well as *P. chlororaphis* strain 30-84. Neither the negative control *E. coli* strain DH5α nor any of the remaining strains produced detectable AHL signals with this assay.

Evaluation of plant protection

We focused our analysis on strain *P. chlororaphis* M71 since *P. aeruginosa* can be a potential human pathogen. Strain M71 was evaluated for the protection of tomato plantlets in seed assays and greenhouse trials. In seed assays, plantlets only treated with the bacterium showed a bacterial population density of 10^6 cfu g^{-1} of roots, while a population density up to 10^7 cfu g^{-1} of roots was detected on roots treated with both the bacterium and the *Forl* pathogen (Table 3). This difference was not detected in greenhouse trials. Disease incidence, assessed as the percentage of dead plantlets was 93% (seed assays) and 83% (greenhouse trials) for *Forl* inoculated plantlets. Application of strain M71 drastically reduced plant mortality in seed assays and almost halved the percentage of diseased plants in greenhouse trials (Table 3).

Discussion

The genus *Pseudomonas* includes several species interesting for their ability to protect plants against pathogenic microorganisms. The biocontrol capability of many bacterial strains belonging to this group has been the main topic in a consistent number of scientific papers (Weller, 2007), and some fluorescent pseudomonads have already been commercialized as biopesticides (i.e. Cedomon, BioAgri, Uppsala, Sweden)). Strain M71, a new fluorescent pseudomonad possessing biocontrol potential, was identified and characterized in this work.

The Biolog Identification System identified the bacterial strain as a member of the *P. chlororaphis* species. Additionally, the phylogenetic analyses of *recA* included strain M71 in the cluster containing the species *P. chlororaphis*, *P. aureofaciens* and *P. aurantiaca*. Since *P. aureofaciens* and *P. aurantiaca* have been reclassified as heterotypic synonyms of *P. chlororaphis* (Johnson and Palleroni, 1989; Peix *et al*., 2007), the strain M71 was allocated in *P. chlororaphis*.

This is the first time that the phylogenetic analyses of *recA* has been coupled with a physiological test for the identification of fluorescent pseudomonads. In recent years, however, sequences of conserved genes (such as *recA*) other than 16S rRNA were proposed for the identification of members of the *Pseudomonas* genus and were considered to be as reliable as classical analyses based on 16S rRNA (Yamamoto *et al*., 2000; Hilario *et al*., 2004). In most of the cases, results from the Biolog system were supported by the phylogenetic analysis of *recA*, thus, the phylogenetic classification of the *Pseudomonas* strains into the five clusters matched the results of physiological identifi-

Table 3. Ability of *Pseudomonas chlororaphis* strain M71 to protect tomato seedlings assessed by seed assays and greenhouse trials. Treatments with the same letters are not statistically different (Tuckey's test, $P<0.05$).

Treatments	Seed assays		Greenhouse trials	
	Disease incidence (%) [a]	Bacterial cell density [b]	Disease incidence (%)	Bacterial cell density
Tomato	0 b	0	0 c	0
Tomato + M71	0 b	2.92×10^6	0 c	9.12×10^5
Tomato + M71+ Forl F55 NA	7 b	1.19×10^7	43 b	6.42×10^5
Tomato + Forl F55 NA	93 a	0	83 a	0

[a] Disease incidence is expressed as the percentage of dead plants on the total of emerged plants.
[b] Bacterial cell density on roots of tomato plants is expressed as cfu g^{-1} of roots.

cation. A wide number of strains must be further tested, in order to fully validate this procedure for the identification of bacterial strains belonging to *Pseudomonas*.

Strain M71 was particularly effective in controlling *F. oxysporum* f. sp. *radicis-lycopersici* both in a seed assay and greenhouse trials. Following the bacterization of tomato seeds, M71 actively colonized tomato plant roots reaching a population level able to control pathogenic activity of the fungus.

Strain M71 produced secondary metabolites with antibiotic activity as shown by the total inhibition of *R. solani* growth *in vitro*. The occurrence of conserved regions of the PHZ and 2,4-DAPG operons involved in the production of two antibiotics in *Pseudomonas* strains was analyzed by PCR. *Pseudomonas chlororaphis* strain M71 showed a 1,000 bp amplicon sharing a high sequence similarity to the *phzF-phzA* region involved in PHZ biosynthesis in *P. chlororaphis* strain 30-84. Biosynthesis of 2,4-DAPG and PHZ is known as the major mechanism by which *Pseudomonas* strains effectively control *Gaeumannomyces graminis* var. *tritici* and *T. basicola* (Thomashow *et al*., 1990; Laville *et al*., 1992).

Furthermore, similarly to strain 30-84, *P. chlororaphis* strain M71 was capable of producing AHL molecules recognized by the two biosensor strains *A. tumefaciens* NT1 (pZLR4) and *C. violaceum* CV026. In strain 30-84, the biosynthesis of PHZ is positively controlled by intraspecific and interspecific communication systems based on the production of small signal molecules belonging to the AHL family (Wood and Pierson, 1996). In strain M71, the production of these signals is correlated with the presence of at least part of the PHZ operon (Wood and Pierson, 1996; Chin-A-Woeng *et al*., 1998). Further investigation is required to assess if *quorum sensing* regulates PHZ biosynthesis in strain M71 too; moreover it will be interesting to determine whether AHL-based *quorum sensing* is conserved across bacterial strains belonging to *P. chlororaphis*.

Fusarium oxysporum f. sp. *radicis-lycopersici* was strongly controlled by strain M71 in seed assays and greenhouse trials where a drastic reduction of tomato plant mortality was observed. The high level of biocontrol activity achieved with this strain is probably due to the combination of different antagonistic mechanisms such as production of antibiotics, proteases, siderophores and AHL molecules. These make *P. chlororaphis* strain M71 a promising new antagonist exploitable for the control of several plant pathogenic fungi.

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Literature cited

Cha C., P. Gao, Y.C. Chen, P.D. Shaw, S.K. Farrand, 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Molecular Plant Microbe Interactions* 11, 1119–1129.

Chin-A-Woeng T.F.C., G.V. Bloemberg, A.J. van der Bij, K.M.G.M. van der Drift, J. Schripsema, B. Kroon, R.J. Scheffer, C. Keel, P.A.H.M. Bakker, H.V. Tichy, F.J. de Bruijn, J.E. Thomas-Oates, B.J.J. Lugtenberg, 1998. Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL 1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Molecular Plant Microbe Interactions* 11, 1069–1077.

Chin-A-Woeng T.F.C., D. van der Broek, G. de Voer, K.M.G.M. van der Drift, S. Tuinmann, J.E. Thomas-Oates, B.J.J. Lugtenberg, 2001. Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1391 is regulated by multiple factors secreted into the growth medium. *Molecular Plant Microbe Interactions* 14, 969–979.

Duffy B. K. and G. Defago, 1997. Zinc improves biocontrol of *Fusarium* crown and root rot of tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic biosynthesis. *Phytopathology* 87, 1250–1257.

Felsenstein J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.

Fuqua W.C., S.C. Winans, E.P. Greenberg, 1994, Quorum Sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology* 176, 269–275.

Haas D. and G. Defago, 2005. Biological control of soil-

borne pathogens by fluorescent pseudomonads. *Nature Review Microbiology* 3, 307–319.

Hilario E., T.R. Buckley, J.M. Young, 2004. Improved resolution on the phylogenetic relationships among *Pseudomonas* by the combined analysis of *atpD*, *carA*, *recA* and 16S rDNA. *Antonie Van Leeuwenhoek* 86, 51–64.

Johnson J.L. and N. Palleroni, 1989. Deoxyribonucleic acid similarities among *Pseudomonas* species. *International Journal of Systematic Bacteriology* 39, 230–235.

Kimura M., 1983. *The Neutral Theory of Molecular Evolution*. Cambridge University Press, Cambridge, UK.

Kumar S., K. Tamura, M. Nei, 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* 5, 150–163.

Laville J., C. Voisard, C. Keel, M. Maurhofer, G. Défago, D. Haas, 1992. Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proceedings of the National Academy of Science USA* 89, 1562–1566.

McClean K.H., M.K. Winson, L. Fish, 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* 143, 3703–3711.

Peix A., A. Valverde, R. Rivas, J.M. Igual, M.H. Ramırez-Bahena, P.F. Mateos, I. Santa-Regina, C. Rodrıguez-Barrueco, E. Martınez-Molina and E. Velazquez, 2007. Reclassification of *Pseudomonas aurantiaca* as a synonym of *Pseudomonas chlororaphis* and proposal of three subspecies, *P. chlororaphis* subsp. *chlororaphis* subsp. nov., *P. chlororaphis* subsp. *aureofaciens* subsp. nov., comb. nov. and *P. chlororaphis* subsp. *aurantiaca* subsp. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 57, 1286–1290

Pierson III L.S., V.D. Kepenne, D.W. Wood, 1994. Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30–84 is regulated by PhzR in response to cell density. *Journal of Bacteriology* 176, 3966–3974.

Puopolo G., A. Raio, A. Zoina, 2010. Identification and characterization of *Lysobacter capsici* strain PG4: a new health-promoting rhizobacterium. *Journal of Plant Pathology* 92, 159–166.

Raaijmakers J.M., D.M. Weller, L.S. Thomashow, 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Applied and Environmental Microbiology* 63, 881–887.

Raaijmakers J.M. and D.M. Weller, 1998. Natural plant protection by 2,4-diacetylphloroglucinol producing *Pseudomonas* spp. in take-all decline soils. *Molecular Plant Microbe Interactions* 11, 144–152.

Saitou N. and M. Nei, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406–425.

Shanmugaiah V., N. Mathivanan and B. Varghese, 2010. Purification, crystal structure and antimicrobial activity of phenazine-1-carboxamide produced by a growth-promoting biocontrol bacterium, *Pseudomonas aeruginosa* MML2212. *Journal of Applied Microbiology* 108, 703–711

Steindler L. and V. Venturi, 2007. Detection of quorum-sensing N-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiology Letters* 266, 1–9.

Stutz E.W. and G. Defago, 1986. Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. *Phytopathology* 76, 181–185.

Thomashow L.S., D.M. Weller, R.F. Bonsall, L.S. Pierson III, 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. *Applied and Environmental Microbiology* 56, 908–912.

Thompson J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876–4882.

Weller D.M., 2007. *Pseudomonas* biocontrol agents of soil-borne pathogens: looking back over 30 years. *Phytopathology* 97, 250–256.

Wood D.W. and L.S. Pierson III, 1996. The *phzI* gene of *Pseudomonas aureofaciens* 30–84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* 168, 49–53.

Yamamoto S., H. Kasai, D.L. Arnold, R.W. Jackson, A. Vivian, S. Harayama, 2000. Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. *Microbiology* 146, 2385–2394.

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