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Functional Characterization of Antagonistic Fluorescent Pseudomonads Associated with Rhizospheric Soil of Rice (*Oryza sativa* L.)

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Abstract Antagonistic fluorescent pseudomonads isolated from rhizospheric soil of rice were characterized by 16S rRNA amplicon and fatty acid methyl ester (FAME) analyses. Antagonistic isolates were grown in the fermentation media, and production of antibiotics was confirmed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Production of fungal cell-wall-degrading enzymes such as protease, cellulase, pectinase, and chitinase was determined. Dendrogram based on the major and differentiating fatty acids resulted into 5 clusters, *viz.*, cluster I (*P. pseudoalcaligenes* group), cluster II (*P. plecoglossicida* group), cluster III (*P. fluorescens* group), cluster IV (*P. aeruginosa* group), and cluster V (*P. putida* group). Characteristic presence of high relative proportions of cyclopropane (17:0 CYCLO w7c) was observed in antagonistic bacteria. Data revealed biodiversity among antagonistic fluorescent pseudomonads associated with the rice rhizosphere. Results presented in this study will help to identify the antagonistic isolates and to determine their mechanisms that mediate antagonism against fungal pathogens of rice.

Keywords: Antagonistic fluorescent pseudomonads, antibiotics, FAME, cyclopropane, dendrogram, 16S rRNA

Rice (*Oryza sativa* L.) is the most important staple food crop of the world. After the introduction of semi-dwarf cultivars of rice, fungal diseases of rice became a hindrance to rice production in all rice-growing countries. Chemical applications, cultural practices, and use of resistant cultivars are routine methods for fungal disease control. Resistant cultivars are not available for every disease, and cost-effective cultural practices are not always feasible. Moreover, available chemical fungicides are often expensive and have adverse effects on human health. Indiscriminate use of chemical fungicides is known to be hazardous to the environment and is lethal to other beneficial rhizosphere bacteria. At this juncture, environment-friendly control of fungal pathogens is a pressing need for sustainable agriculture [8].

Antagonistic bacteria that are target-specific, eco-friendly, and, most importantly, capable of colonizing in the inoculated environment are in the forefront for the effective biocontrol of fungal pathogens. Fluorescent pseudomonads are distributed widely in temperate as well as in tropical soils and often predominate among bacteria of plant rhizosphere. Specific strains of fluorescent pseudomonads have the potential to suppress plant pathogens, enhance plant growth, and participate in carbon and nitrogen cycling in nature [1, 16]. Antagonistic fluorescent pseudomonads are known to produce an array of metabolites such as phenazines [10, 17, 24], pyrrole-type compounds, and polyketides [2, 7]. Specific metabolites may elicit defense reactions of the host plant [27]. Therefore, the role of fluorescent pseudomonads in agriculture has been a matter of interest.

Characterization of antagonistic bacteria is important for registration, patenting, recognition, and quality checking. Antagonistic isolates of fluorescent pseudomonads used for the biological control of rice diseases have been reported [15, 20]. To our knowledge, there is no report concerning fatty acid methyl ester (FAME) analysis-based detection and differentiation of antagonistic fluorescent pseudomonads associated with rice rhizospheric soil. Therefore, the present investigation was aimed to detect antagonistic fluorescent pseudomonad isolates and gain insight on the variability of isolates by employing 16S rRNA and FAME analyses and to study their mechanisms of antagonism.

Materials and Methods

Microbial Cultures

Standard bacterial strains, *Pseudomonas fluorescens* Pf5, *P. fluorescens* 2-79, and *P. aeruginosa* PAO1 were supplied

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by Linda S. Thomashow (USDA, Washington State University, Pullman, WA, U.S.A.) and *P. fluorescens* CHAO was supplied by Genevieve Defago (Swiss Federal Institute of Technology, Zurich, Switzerland). *P. stutzeri* MTCC 863 was obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India. Rice fungal pathogens, *Rhizoctonia solani* RSR1 (sheath blight), *Magnaporthe grisea* MGS (blast), and *Sarocladium oryzae* SONS (sheath rot) were obtained from the Microbial Culture Collection (MCC), Department of Biotechnology, Pondicherry University, Pondicherry, India. Microbial cultures were maintained at the Department of Biotechnology, Pondicherry University, Pondicherry.

Isolation of Fluorescent Pseudomonads

Rice roots with adhering soil were collected from a rice field located at Pondicherry. The soil was sand clay loam and its characteristics were as follows: 7.1 pH, 67.50 μg/g N, 3.3 μg/g P, 5.17 μg/g K, and 0.2 mmhos/cm electrical conductivity (EC). Fluorescent pseudomonads were isolated as described earlier [21]. Briefly, to quantify the total heterotrophic bacterial population, the rhizospheric soil suspension was obtained by shaking 10 g of roots with adhering soil in 90 ml of 0.1 M $MgSO_4 \cdot 7H_2O$ buffer for 10 min. Ten-fold dilutions of $MgSO_4 \cdot 7H_2O$ extracts from rhizospheric soils were plated onto King's B (KB) agar medium [13]. After incubation at 25°C for 48 h, bacterial colony counts were made, fluorescent colonies were identified under ultraviolet (UV) light at 360 nm, and single colonies were further streaked onto KB agar for obtaining pure cultures.

Fungal Inhibition Bioassays

Bacteria were tested for *in vitro* antagonism towards fungal pathogens by following standard co-inoculation techniques on potato dextrose agar (PDA) [20]. Briefly, bacterial plugs (6 mm diameter) were removed from a 48 h culture. The plugs were transferred to the center of PDA plates, which had been inoculated with fungal spore suspension (10^6 conidia/ml). Assay plates were incubated at 28°C for 3 days and growth-inhibition that appeared around the bacterial plugs was measured.

Production of Fungal Cell-Wall-Degrading Enzymes

Production of protease was determined using skim milk agar on the basis of proteolytic activity [20]. Cellulase and pectinase were determined on M9 agar medium amended with cellulose and pectin, respectively [3]. Chitinase activity was determined by plating bacteria on chitin agar as previously described [18].

Production of Hydrogen Cyanide (HCN)

Test for the production of HCN was carried out as described earlier [5, 24]. Briefly, isolates were streaked onto KB agar plates supplemented with glycine (4.4 g/l) to screen cyanide production. After this, the Petri dishes were inverted and a piece of filter paper impregnated with 0.5% picric acid (yellow) and 2% sodium carbonate was placed on the lid. Petri dishes were sealed with parafilm and incubated at 28°C for 96 h. Discoloration of the filter paper to orange to brown after incubation indicates microbial production of cyanide.

Production of Antifungal Metabolites

Antibiotics were extracted as described earlier [3, 16]. Productions of antibiotics were tested by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) [5, 11, 25]. TLC was carried out on silica gel G60 (20×20 cm; 0.25 mm thick; Selecto Scientific, GA, U.S.A.). The plates were activated at 110°C for 30 min, cooled, and spotted with ethanol solution containing standard antibiotics (0.5 μg) and 20 μl of extract. Separation was performed with chloroform-methanol (9:1 v/v) for phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (DAPG), or chloroform-acetone (9:1 v/v) for pyoluteorin (PLT) and pyrrolnitrin (PRN). The corresponding spots by PCA, DAPG were detected by UV at 254 nm [25]. PLT spots were detected by spraying with an aqueous 0.5% (w/v) Fast Blue RR salt solution, and the PRN spots were detected by spraying the TLC plates with 2% *p*-dimethylaminobenzaldehyde dissolved in the ethanol-sulfuric acid (1:1 v/v) [5]. Production of antibiotics PCA and DAPG was also verified by analytical HPLC methods as described earlier [11]. Purified extracts were resuspended in 1 ml of methanol (HPLC grade) and subjected to C_{18} reverse-phase HPLC (Phenomenex Luna, 250×10 mm) with 30-μl injection volumes. The solvent conditions included a flow rate of 0.7 ml/min with acetonitrile and water (both containing 0.1% trifluroacetic acid) in a 30–70% linear gradient for PCA. The solvent conditions included a flow rate of 2 ml/min with 80% (v/v) acetonitrile in water for DAPG. HPLC gradient profiles were monitored at 257 nm for PCA and 270 nm for DAPG using a UV detector 10 AVP (Shimadzu, Kyoto, Japan).

Fatty Acid Methyl Ester (FAME) Analyses

For FAME analyses, bacterial cultures were grown on tryptic soy agar (TSA) in triplicate and incubated for 24 h at 28°C. Cells (50 mg wet weight) were scraped and suspended in 1 ml of saponification reagent in a screw-cap test tube and vortexed for 10 s. The tube was then placed in a water bath at 100°C for 25 min, cooled to room temperature, and 2 ml of methylation reagent was added. The mixture was then vortexed for 10 s, placed in a water bath at 80°C for 10 min, and rapidly cooled by placing on ice. Then, extraction buffer (1.25 ml) was added and mixed well for 10 min. The aqueous lower layer was separated and discarded. To the upper organic phase, 3 ml of base

wash reagent was added and mixed well for 5 min. The mixture was then centrifuged at 3,000 rpm for 5 min. The upper solvent phase was removed and analyzed by gas-liquid chromatography (Hewlett-Packard 6890, Avondale, U.S.A.) using capillary column Ultra 2-HP (cross-linked 5% phenyl-methyl silicone, 25 m, 0.22 mm; film thickness, 0.33 μm) and hydrogen as the carrier gas. FAME compounds were detected by a flame ionization detector (FID) and identified using the Microbial Identification Software (Sherlock aerobe method and TSBA40 Library Version 4.5) developed by MIDI Inc., Newark, DE, U.S.A. Data were converted to binary code and distance matrix was calculated by using pairwise co-efficient of similarity (Dice). Cluster analysis was done by using the unweighted pair group method with mathematical averages (UPGMA) algorithm using the NTSYSpc2 (Version 2.02a, Exeter software, New York, U.S.A.) numerical taxonomy and multivariate analysis system.

16S rRNA Amplification, Sequencing, and Phylogenetic Tree Analysis

Amplification of the 16S rRNA gene was performed from the genomic DNA of antagonistic bacteria as described earlier, using universal primers fD1 and rP2 [29]. PCR products were purified using the Microcon PCR centrifugal filter device (Millipore Corporation, Bedford, U.S.A.) and sequenced with an automated DNA sequencer with specific primers, using the facility at Macrogen Inc (Seoul, Korea). The 16S rRNA sequences were subjected to Blast search from the NCBI database for bacterial strain identification. The reference sequences required for comparison were obtained from the EMBL database using the site http://www.ncbi.nlm.nih.gov/Genbank. All the 16S rRNA sequences of antagonistic fluorescent pseudomonads were aligned using the multiple sequence alignment program CLUSTAL V developed by Higgins *et al.* [9]. The aligned sequences were then checked for gaps manually, arranged in a block of 250 bp in each row, and saved as molecular evolutionary genetics analysis (MEGA) format in the software MEGA v2.1. The pairwise evolutionary distances were computed using the Kimura 2-parameter model as developed by Kimura [12]. To obtain the confidence values, the original data set was resampled 1,000 times using the bootstrap analysis method. The bootstrapped data set was used directly for constructing the phylogenetic tree using the MEGA program or used for calculating the multiple

Table 1. Mechanism of antagonism of fluorescent pseudomonads against rice fungal pathogens.

Isolates	Inhibitory fungi of rice	Mechanism of antagonism	
		Antifungal metabolites	Cell-wall-degrading enzymes
P1	M.g, S.o	ND	ND
P2	M.g, S.o	DAPG, PRN, PLT	ND
P3	M.g, S.o	DAPG, PRN, PLT	PRO, PEC
P4	M.g, S.o	HCN	ND
P5	M.g, S.o	HCN, DAPG, PRN, PLT	PEC
P6*	M.g, S.o	ND	PRO
P7	M.g, S.o, R.s	HCN, DAPG, PRN, PLT	PRO, CELL, CHI
P8	M.g, S.o	HCN, DAPG, PRN, PLT	PRO
P9	M.g, S.o	PLT	PRO
P10	M.g, S.o, R.s	HCN, DAPG	CHI
P11	M.g, S.o, R.s	ND	PRO, CELL, CHI
P12	M.g, S.o, R.s	ND	PRO
P13	M.g, S.o, R.s	ND	PRO
P14	M.g, S.o	HCN, DAPG, PRN, PLT	ND
P15	M.g, S.o	PCA	ND
P16	M.g, S.o	ND	CELL
P17	M.g, S.o	ND	CELL
P18	M.g, S.o	HCN, DAPG, PRN, PLT	ND
P19	M.g, S.o	PRN, PLT	PRO
P20	M.g, S.o	ND	PRO
P21	M.g, S.o	HCN, DAPG, PRN, PLT	PRO
P22	M.g, S.o	PRN, PLT	CELL
P23	M.g, S.o	ND	ND
P24	M.g, S.o	ND	PRO
P25	M.g, S.o	ND	PRO, PEC

M.g, *Magnaporthe grisea*; S.o, *Sarocladium oryzae*; R.s, *Rhizoctonia solani*; HCN, hydrogen cyanide; DAPG, 2,4-diacetylphloroglucinol; PCA, phenazine-1-carboxylic acid; PRN, pyrrolnitrin; PLT, pyoluteorin; PRO, protease; PEC, pectinase; CELL, cellulase; CHI, chitinase; *Close relative of *Pseudomonas* (formerly, *P. maltophilia*)

distance matrixes. The multiple distance matrix obtained was then used to construct phylogenetic trees using the neighbor-joining (NJ) method of Saitou and Nei [19]. All these analyses were performed using the MEGA v2.1 [14].

Nucleotide Sequence Accession Numbers

Accession numbers of the 16S rRNA nucleotide sequences of the isolates submitted to GenBank are DQ201392-DQ201416.

Results

Isolation and Screening of Antagonistic Fluorescent Pseudomonad Bacteria

Based on the heterotrophic plate count, the total population of aerobic bacteria, and fluorescent bacteria associated with rice rhizosphere was 2.6×10^6 and 6.4×10^2 CFU/g soil, respectively. Among 750 bacterial isolates of rice rhizosphere, 25 isolates showed antifungal activity towards phytopathogenic fungi used in the study. Antagonistic bacterial isolates induced growth-free inhibition zones (diameter) ranging from 4–35 mm.

Production of Antifungal Metabolites

Antifungal metabolites such as DAPG (yellowish white), PCA (greenish yellow), PRN (light yellow), and PLT (yellowish white) were extracted from the fermentation cultures. TLC and HPLC analyses confirmed the production of PCA, DAPG, PLT, and PRN by isolates (Table 1). The retardation factor (Rf) values were 0.77 for DAPG, 0.53 for PCA, 0.80 for PRN, and 0.50 for PLT as determined by comigration with pure standards (Fig. 3). DAPG was detected at 270 nm and its retention time was 10.77 min. PCA was detected at 257 nm and its retention time was 4.94 min (Fig. 4). A total of 8 isolates produced HCN (Table 1).

Table 2. Grouping of antagonistic fluorescent pseudomonads on the basis of major and differentiating fatty acids as determined by FAME analyses.

Fatty acid	Groups (Isolates)				
	Group I	Group II	Group III	Group IV	Group V
	(P1, 4)	(P20)	(P2, 3, 5, 7–9, 14, 16–19, 21–23)	(P10, 11, 13)	(P12, 15, 24, 25)
$C_{11:0}$	+	-	-	-	-
$C_{11:3\ OH}$	+	-	-	-	-
$C_{12:0\ 2OH}$	-	+	+	+	+
$C_{14:0\ ISO}$	-	-	+	-	-
$C_{15:1\ w8c}$	+	-	-	-	-
$C_{16:0\ ISO}$	+	-	-	-	-
$C_{16:0\ OH}$	-	-	+	-	-
$C_{17:1\ w6c}$	-	-	+	-	-
$C_{19:0\ 10}$	-	-	+	-	+
$C_{20:2\ w9c}$	-	+	-	-	-

Group I, *P. pseudoalcaligenes*; group II, *P. plecoglossicida*; group III, *P. fluorescens*; group IV, *P. aeruginosa*; group V, *P. putida*. $C_{11:0}$, Hendecanoic acid; $C_{11:3\ OH}$, Hendecanoic acid, 3-hydroxy-; $C_{12:0\ 2OH}$, Decanoic acid, 2-hydroxy-; $C_{14:0\ ISO}$, Tridecanoic acid, 12-methyl-; $C_{15:1\ w8c}$, cis-delta8-Pentadecanoic acid; $C_{16:0\ ISO}$, Pentadecanoic acid, 14-methyl-; $C_{17:0\ w6c}$, cis-delta6-Heptadecanoic acid; $C_{19:0\ 10}$ Nonadecanoic acid; $C_{20:2\ w9c}$, cis-delta9-Ecosanoic acid.

Production of Cell-Wall-Degrading Enzymes

A total of 13 isolates produced protease, 5 isolates produced cellulase, 3 isolates produced pectinase, and 3 isolates produced chitinase (Table 1).

Fatty Acid Methyl Esters (FAME) Analyses

In FAME analyses, the proportion of fatty acid in each species isolate was identified as a variable. By comparison with the commercially available database (MIDI, Newark, DE, U.S.A.), isolates were identified as follows: P1 as *Pseudomonas pseudoalcaligenes*; P3 and P8 as *P. fluorescens*; P10, P11, and P13 as *P. aeruginosa*; P20 as *P. plecoglossicida*; P12, P15, and P25 as *P. putida* biotype A; and P24 as *P. putida* biotype B (Table 3). Isolate P6 was identified as a non-pseudomonad bacterium, *Stenotrophomonas maltophilia* (formerly, *P. maltophilia*).

On the basis of major and differentiating fatty acids, all antagonistic fluorescent pseudomonad isolates were grouped into five major groups (Table 2, Fig. 1). Group I showed the presence of hendecanoic acid ($C_{11:0}$), hendecanoic acid, 3-hydroxy- ($C_{11:3\ OH}$), cis-delta8-pentadecanoic acid ($C_{15:1\ w8c}$), and pentadecanoic acid, 14-methyl- ($C_{16:0\ ISO}$); group II contained fatty acids, decanoic acid, 2-hydroxy- ($C_{12:0\ 2OH}$) and cis-delta9-ecosanoic acid ($C_{20:2\ w9c}$); and group III showed the characteristic presence of decanoic acid, 2-hydroxy-($C_{12:0\ 2OH}$), tridecanoic acid, 12-methyl-($C_{14:0\ ISO}$), pentadecanoic acid, 14-methyl- ($C_{16:0\ 3OH}$), cis-delta6-heptadecanoic acid ($C_{17:1\ w6c}$), and nondecanoic acid ($C_{19:0\ 10\ methyl}$). Whereas

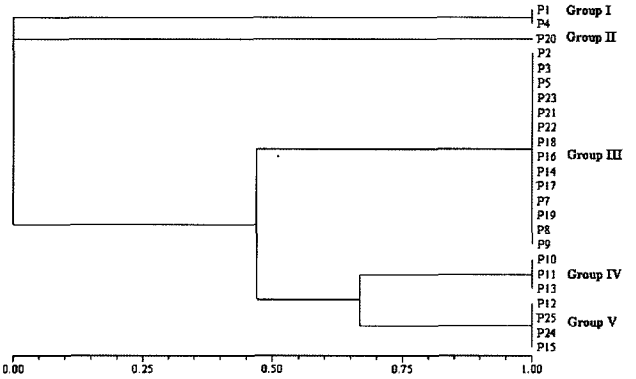

Fig. 1. Dendrogram of antagonistic fluorescent pseudomonads based on their major and differentiating fatty acids as determined by FAME analysis.

Group I, *P. pseudoalcaligenes*; group II, *P. plecoglossicida*; group III, *P. fluorescens*; group IV, *P. aeruginosa*; group V, *P. putida*.

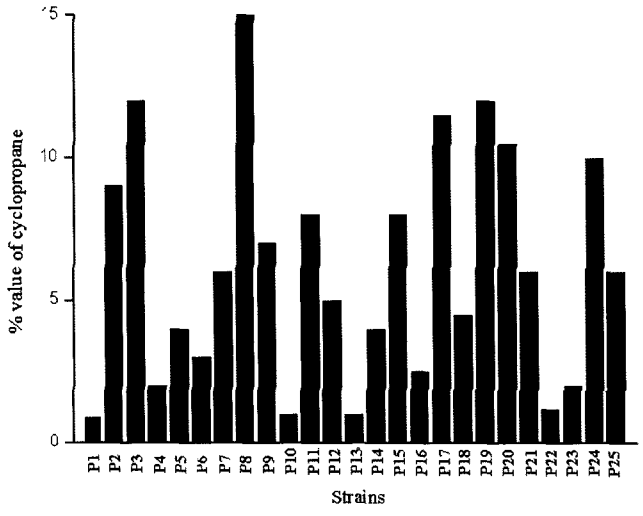

Fig. 2. Proportion of cyclopropane in the antagonistic fluorescent pseudomonad isolates and *Stenotrophomonas maltophilia.*

group IV showed the presence of decanoic acid, 2-hydroxy-($C_{12:0\ 2OH}$), group V showed the presence of decanoic acid, 2-hydroxy-($C_{12:0\ 2OH}$) as well as nonadecanoic acid ($C_{19:0\ 10\ methyl}$). Characteristic presence of high relative proportions (0.7–14%) of cyclopropane (17:0 CYCLO w7c) was observed in all antagonistic fluorescent pseudomonad isolates (Fig. 2). The non-antagonistic control isolates did not show a detectable level of cyclopropane.

16S rRNA Amplification, Sequencing, and Phylogenetic Tree Analysis

Fluorescent pseudomonad species such as *P. fluorescens*, *P. aeruginosa*, *P. putida*, *P. pseudoalcaligenes*, *P. plecoglossicida*, and the non-pseudomonad bacterium *S. maltophilia* (formerly, *P. maltophilia*) were identified on the basis of ribosomal operon (16S rRNA) gene homology (92–99%) (Table 3). Out of 25 isolates, 14 isolates (P2, P3, P5, P7-P9, P14, P16-P19, P21-P23) belonged to *P. fluorescens*, 4 isolates (P12, P15, P24, P25) belonged to *P. putida*, 3 isolates (P10, P11, P13) belonged to *P. aeruginosa*, 2 isolates (P1, P4) belonged to *P. pseudoalcaligenes*, 1 isolate (P20) belonged to *P. plecoglossicida*, and 1 isolate (P6) was identified as a non-pseudomonad bacterium, *S. maltophilia* (formerly, *P. maltophilia*).

Table 3. Taxonomic affiliation and GenBank accession number of antagonistic fluorescent pseudomonad isolates based on the similarity of FAME and 16S rDNA sequences.

Isolates	GenBank accession number	Closest hits database	Similarity from database (%)	
			NCBI	MIDI
P1	DQ201392	*Pseudomonas pseudoalcaligenes*	97	98
P2	DQ201393	*Pseudomonas fluorescens*	99	ND
P3	DQ201394	*Pseudomonas fluorescens*	99	98
P4	DQ201395	*Pseudomonas pseudoalcaligenes*	98	ND
P5	DQ201396	*Pseudomonas fluorescens*	97	ND
P6*	DQ201397	*Stenotrophomonas maltophilia*	99	99
P7	DQ201398	*Pseudomonas fluorescens*	97	ND
P8	DQ201399	*Pseudomonas fluorescens*	96	98
P9	DQ201400	*Pseudomonas fluorescens*	96	ND
P10	DQ201401	*Pseudomonas aeruginosa*	99	99
P11	DQ201402	*Pseudomonas aeruginosa*	98	99
P12	DQ201403	*Pseudomonas putida*	99	98
P13	DQ201404	*Pseudomonas aeruginosa*	99	98
P14	DQ201405	*Pseudomonas fluorescens*	99	ND
P15	DQ201406	*Pseudomonas putida*	99	98
P16	DQ201407	*Pseudomonas fluorescens*	99	ND
P17	DQ201408	*Pseudomonas fluorescens*	99	98
P18	DQ201409	*Pseudomonas fluorescens*	92	ND
P19	DQ201410	*Pseudomonas fluorescens*	98	ND
P20	DQ201411	*Pseudomonas plecoglossicida*	98	ND
P21	DQ201412	*Pseudomonas fluorescens*	98	98
P22	DQ201413	*Pseudomonas fluorescens*	98	ND
P23	DQ201414	*Pseudomonas fluorescens*	99	98
P24	DQ201415	*Pseudomonas putida*	98	98
P25	DQ201416	*Pseudomonas putida*	98	98

ND, Not determined.
*Close relative of *Pseudomonas* (formerly, *P. maltophilia*).

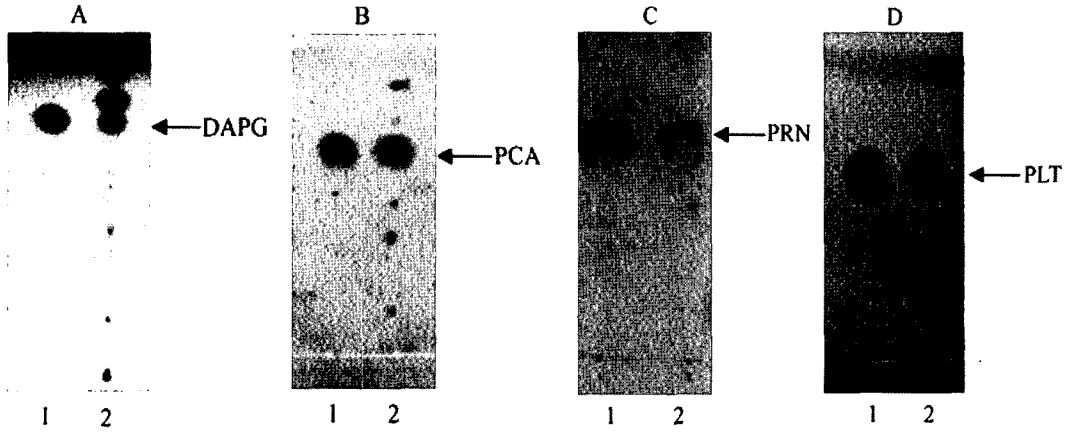

Fig. 3. TLC profiles of antifungal metabolites produced by the antagonistic fluorescent pseudomonads.
A. 2, 4-Diacetylphloroglucinol (DAPG), lane 1, Standard (0.5 μg); lane 2, DAPG extract of P10 (20 μl). **B**. Phenzine-1-carboxylic acid (PCA), lane 1, standard (0.5 μg); lane 2, PCA extract of P7 (20 μl). **C**. Pyrrolnitrin (PRN), lane 1, standard (0.5 μg); lane 2, PRN extract of P7 (20 μl). **D**. Pyoluteorin (PLT), lane 1, standard (0.5 μg); lane 2, PLT extract of P2 (20 μl).

Phylogenetic analyses of 24 antagonistic fluorescent pseudomonad isolates were divided into 3 major clusters (Fig. 5). The *P. putida* cluster consisted of 4 isolates of *P. putida* (P12, P15, P24, P25), 2 isolates of *P. pseudoalcaligenes* (P1, P4), and 1 isolate of *P. plecoglossicida* (P20) along with the reference isolates *P. pseudoalcaligenes* (AB1098888, AB021379, AJ984813), *P. plecoglossicida* (AB009457, DQ095898), and *P. putida* (DQ141542). The *P. aeruginosa* cluster consisted of 3 isolates of *P. aeruginosa* (P10, P11, P13) along with the reference isolates *P. aeruginosa* (AY792969, DQ115539), *P. denitrificans* (AB021419), *P. pertucinogena* (AB021380), and *P. anguilliseptica* (AB021376). The *P. fluorescens* cluster contained 14 isolates of *P. fluorescens* (P2, P3, P5, P7-P9, P14, P16-P19, P21-P23) along with the reference isolates *P. fluorescens* (D84013, AY196702), *P. marginalis* (AB021401), and *P. libabiensis* (AF057645). The sequences of *S. maltophilia* were treated as the out-group in the phylogenetic tree.

Discussion

In recent years, much attention has been given to the antagonistic activities of fluorescent pseudomonad bacteria from agricultural crop plants. The predominant nature of fluorescent pseudomonads in rhizosphere soils of plants has been reported [21, 22], but the genetic variability and functional characterization of potent antagonistic bacteria

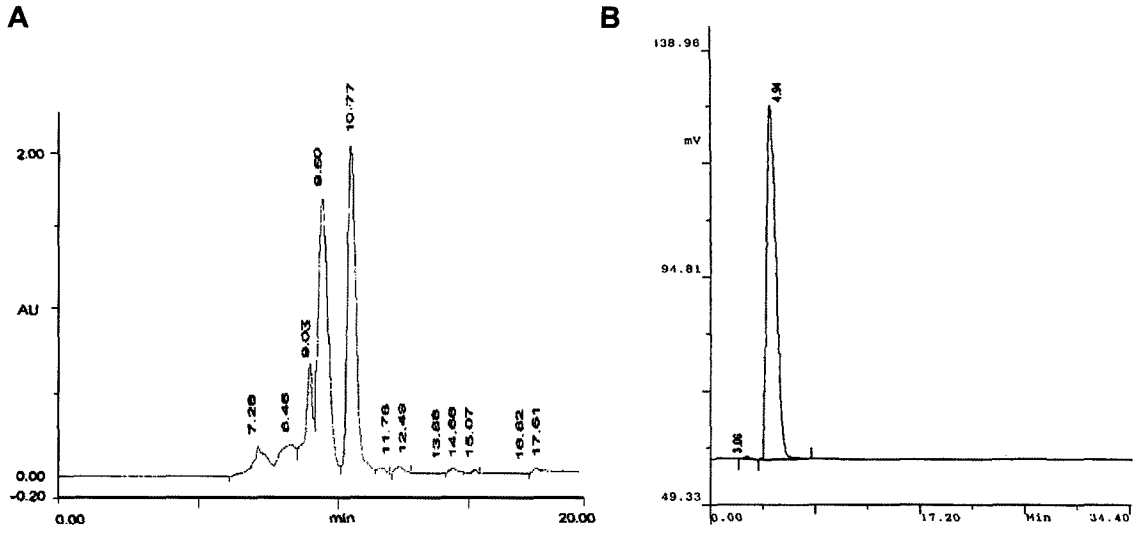

Fig. 4. HPLC chromatogram of antifungal metabolites.
A. DAPG produced by antagonistic fluorescent pseudomonad isolate P7 (retention time 10.77 min). **B**. PCA produced by antagonistic fluorescent pseudomonad isolate P10 (retention time 4.94 min).

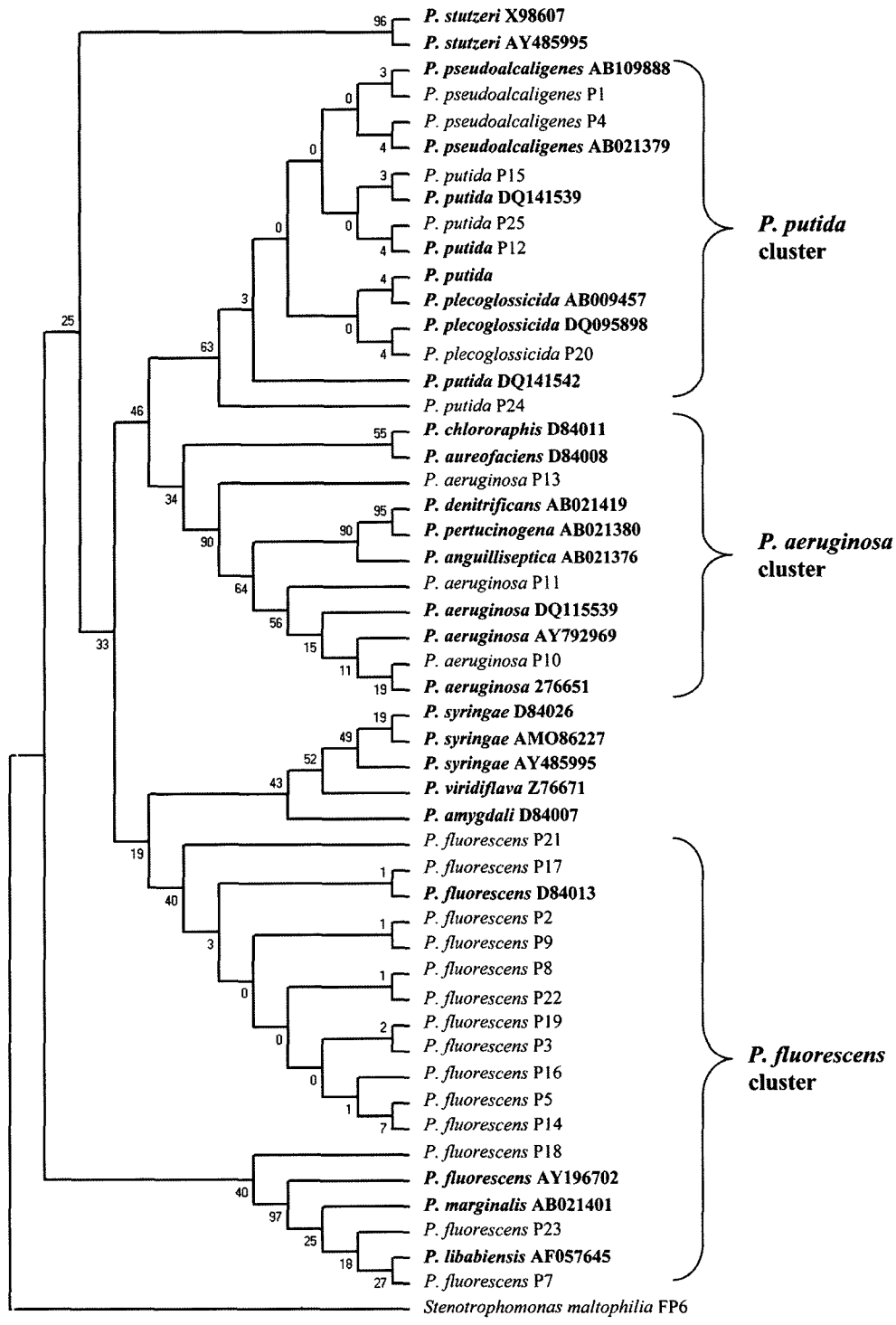

Fig. 5. Phylogenetic tree of 24 antagonistic fluorescent pseudomonad isolates based on the 16S rRNA sequences.
The tree was constructed using the neighbor-joining method. The sequences of *S. maltophilia* were treated as the out-group.

is just beginning to be explored. The role of fluorescent pseudomonads in agriculture has been a matter of interest because of their abundant population in plant rhizosphere and their innate biocontrol properties. The results of the present investigation revealed the biodiversity and predominant nature of *P. fluorescens* among antagonistic fluorescent pseudomonads in the rhizosphere of rice.

Fatty acid profiling of antagonistic fluorescent pseudomonad isolates revealed a considerable degree of diversity. Identification of isolates based on FAME and 16S rRNA

yielded good agreement, except for a few isolates by FAME, due to low-level match. However, FAME profiling resulted into 5 different groups and greatly facilitated the assessment of the extent of interrelatedness among isolates. FAME analyses also facilitated the identification of cyclopropane fatty acid as a FAME marker for antifungal activity in antagonistic fluorescent pseudomonad isolates of rice rhizosphere. As with antifungal metabolites, the production of cyclopropane occurs primarily in the stationary phase of the growth cycle of fluorescent pseudomonads under the control of *rpoS* [7, 28]. Therefore, the level of cyclopropane in antagonistic fluorescent pseudomonad isolates may indicate the overall efficiency of the production of antifungal secondary metabolites controlled by stationary-phase regulators, such the sigma factor σ^S [6]. Our results had good agreement with an earlier report on the utilization of cyclopropane as a trait for the selection of *P. chlororaphis* strain S34/10 as a biocontrol agent [6]. Although cyclopropane is common to fluorescent pseudomonads [26], only antagonistic isolates had detectable quantities. Therefore, identification of isolates for elevated synthesis of cyclopropane could be used as a criterion for the rapid screening of antagonistic fluorescent pseudomonads.

The present study revealed the biodiversity among antagonistic fluorescent pseudomonads of rice rhizospheric soil and identified different species such as *P. pseudoalcaligenes*, *P. fluorescens*, *P. aeruginosa*, *P. putida*, and *P. plecoglossicida* and *S. maltophilia*. These antagonistic bacteria showed the elevated synthesis of cyclopropane fatty acid and exhibited the production of one or more antifungal metabolites and fungal cell-wall-degrading enzymes. The results of the present investigation indicated that FAME analysis can be used as a tool for the detection and differentiation of antagonistic bacteria.

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