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Genetic and Functional Diversity Among the Antagonistic Potential Fluorescent Pseudomonads Isolated from Tea Rhizosphere

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Abstract Twenty-five fluorescent pseudomonads from rhizospheric soil of six tea gardens in four district of Upper Assam, India were isolated and screened for antagonistic activity against fungal pathogens such as *Fusarium oxysporum* f. sp. *raphani* (For), *Fusarium oxysporum* f. sp. *ciceri* (Foc), *Fusarium semitectum* (Fs), and *Rhizoctonia solani* (Rs); and bacterial pathogens—*Staphylococcus aureus* (Sa), *Escherichia coli* (Ec), and *Klebsiella pneumoniae* (Kp). Most of the isolates exhibited strong antagonistic activity against the fungal pathogens and gram-positive bacterium i.e. *Staphylococcus aureus*. Productions of siderophore, salicylic acid (SA), hydrogen cyanide (HCN), and cell wall-degrading enzyme (chitinase) were studied to observe the possible mechanisms of antagonistic activity of the isolates. Correlation between the antagonistic potentiality of some isolates and their levels of production of siderophore, salicylic acid, and hydrogen cyanide was observed. Out of the 25 isolates, antibiotic-coding genes, 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin (PLT) were detected in the isolates, Pf12 and Pf373, respectively. Genetic diversity of these fluorescent pseudomonads were analyzed with reference to four strains of *Pseudomonas fluorescens* NICM 2099^T, *P. aeruginosa* MTCC 2582^T, *P. aureofaciens* NICM 2026^T, and *P. syringae* MTCC 673^T. 16S rDNA-RFLP analysis of these isolates using three tetra cutter restriction enzymes (*Hae*III, *Alu*I and *Msp*I) revealed two distinct clusters. Cluster A comprised only two isolates Pf141 and 24-PfM3, and cluster B comprised 23 isolates along with four reference strains.

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Introduction

Fluorescent pseudomonads are an ecologically important group of soil bacteria that are well accepted as plant growth promoting rhizobacteria (PGPR) [1, 2] and biocontrol agent [3–5]. These bacteria influence plant growth directly or indirectly by preventing the deleterious effect of phytopathogenic organisms [1, 6]. In the last two decades, a renewed interest in biological control of plant diseases in agriculture and horticulture has evolved, partly in response to public concern about the use of hazardous agrochemicals. The study of the root-associated bacteria and their antagonistic potential is important not only for understanding their ecological role in the rhizosphere and the interaction with plants but also for many biotechnological applications. Research endeavor leading to environmental-friendly alternative to protect root diseases is rhizobacterium-mediated biological control, and the mechanisms towards plant pathogen such as fungi and bacteria encompass the competition for nutrients and space, the production of antibiotics, HCN, iron-chelating siderophores, salicylic acid, and the production of fungal cell wall-degrading enzymes [2, 7–9].

In recent years, much attention has been given to study the diversity of fluorescent pseudomonads endowed with their biocontrol and biofertilizing abilities [6, 10]. The predominant nature of fluorescent pseudomonads in rhizospheric soils of plant has been reported [11–13]. Tea is one of the major cash crops in India and is the third largest producer in the world. Moreover, tea is the most widely, consumed beverage in the world [14]. Tea plantations in India are concentrated in Assam (Upper Assam) of North-East India and produce about 400 million kg of tea per year. Moreover, North-East India is the best known for its rich biodiversity, and its untapped bioresources has

been identified as the Indo-Burma Mega Hot Spot by Conservation International [15]. Although, very few workers have reported the diversity of fluorescent pseudomonads in rhizopheric soil of tea in certain parts of India [16, 17], however, no genetic and functional diversity of fluorescent pseudomonads in tea rhizosphere has been explored in North-East India. Moreover, the characterization of antagonistic fluorescent pseudomonads prevalent in an area is essential for the efficient management of diseases and increasing crop productivity. Therefore, the present investigation was aimed to observe (i) the antagonistic potentiality of fluorescent pseudomonads associated with tea rhizopheric soil, (ii) the functional diversity of the isolates, (iii) the genetic diversity through 16S rDNA-RFLP studies, and (iv) the presence of known antibiotic producing genes, 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin (PLT).

Materials and Methods

Soil Sampling

Soil samples were collected from the rhizosphere of tea plants from four districts, viz., Dibrugarh, Sivsagar, Jorhat, and Golaghat of Upper Assam, India (Table 1). The sites were sampled from five different locations in each tea garden along with the root-adhering soil. The soil samples from each tea garden were combined and passed through 2-mm sieve and were kept at 4°C until use.

Isolation of Bacteria

A total of 200 bacterial isolates were isolated from the collected soils in King's B (KB) medium and pure cultures were obtained by repeated streaking on KB medium and screened through UV light ($\lambda = 356$ nm) to identify fluorescent pseudomonads. Thus, 25 isolates were shortlisted on the basis of fluorescent test and kept in 4°C until use (Table 1).

Microbial Strains

Fungal pathogens such as *Fusarium oxysporum* f. sp. *raphani* (FoRN5), *Fusarium oxysporum* f. sp. *ciceri* (FocRs9), *Fusarium semitectum* (FsNJ9), and *Rhizoctonia solani* (RsNJ10) were taken from the Culture Bank of Biotechnology Division, North-East Institute of Science Technology, Jorhat, Assam, India. The bacterial pathogens *Staphylococcus aureus* (MTCC 737^T), *Escherichia coli* (MTCC 739^T), and *Klebsiella pneumoniae* (MTCC 109^T) were obtained through the courtesy of Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India.

Table 1 Antagonistic potential fluorescent pseudomonads isolated from four different tea gardens of Assam

Sl. No.	Isolates	Place of collation
1	PfPMG	Barbarua Tea Garden (BTG), Dibrugarh district
2	Pf146	BTG
3	Pf140	BTG
4	Pf351	BTG
5	Pf125	BTG
6	Pf165	BTG
7	Pf214	BTG
8	Pf87	Khowang Tea Garden (KTG), Dibrugarh district
9	Pf355	KTG
10	Pf245	KTG
11	PfM11	KTG
12	Pf12	Rajmai Tea Garden (RTG), Sivsagar district
13	Pf13	RTG
14	Pf14	RTG
15	PfM31	Chenijan Tea Garden (CTG), Jorhat district
16	Pf197	CTG
17	Pf373	CTG
18	Pf264	CTG
19	Pf238	Kakajan Tea Garden (KaTG), Jorhat district
20	Pf213	KaTG
21	Pf83	KaTG
22	Pf130	Behora Tea Garden (BeTG), Golaghat district
23	Pf141	BeTG
24	PfM3	BeTG
25	Pf59	BeTG

Reference strains of pseudomonads, *Pseudomonas fluorescens* (NICM 2099^T) and *P. aureofaciens* (NICM 2026^T) were obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India; *P. aeruginosa* (MTCC 2582^T) and *P. syringae* (MTCC 673^T) were from IMTECH, Chandigarh.

Phenotypic Characterization

Bacterial isolates along with the reference strains were observed under microscope after gram staining and different biochemical tests such as nitrate reduction, catalase, oxidase, methyl red, starch hydrolysis, lipase production, fluorescent pigments, motility, and sugar (glucose) utilization were carried out as per the method of Bergey's Manual of Determinative Bacteriology (1994) with five replications for each test.

In Vitro Screening for Antimicrobial Activity

Antagonistic activity of the fluorescent isolates were screened towards the fungal pathogens, viz., *F. oxysporum* f. sp. *raphani, F. oxysporum* f. sp. *ciceri, F. semitectum,* and *R. solani* as well as bacterial pathogens, *S. aureus, E. coli,* and *K. pneumoniae* using secondary metabolite of the culture broth. Secondary metabolites were extracted as described earlier [18]. This crude extract was dissolved in 10% dimethyl sulfoxide (DMSO) and used for screening of antimicrobial activity by agar well diffusion method [19]. Zone of inhibition was recorded after 2 days of incubation at 28 ± 1°C for bacterial pathogens and 5 days for the fungal pathogens with five replications.

Screening for Antimicrobial Traits

Siderophore Assay

Siderophore production was quantified as described earlier [20]. The absorbance for dihydroxyl phenols was read in a spectrophotometer at 700 nm. A standard curve was drawn with dihydroxy benzoic acid, and the quantity of siderophore synthesized was expressed as µmol of benzoic acid/ml of culture filtrate, and five replications were made for each experiment.

Assay for Salicylic Acid (SA) Production

The bacterial strains were grown in plastic tube containing 20 ml King's B (KB) broth and incubated at 28 ± 2°C for 28 h. The culture was centrifuged at 2800×*g* for 20 min at 4°C, and then the supernatant was acidified to pH 2 using 1 N HCl. The solution was filtered through nylon membrane under vacuum and partitioned twice with 2 ml $CHCl_3$, and finally dried under nitrogen stream at 40°C. Each sample was re-suspended in 1 ml of 23% methanol in 20 mM sodium acetate buffer (pH 5), and then the samples were analyzed (µg/ml bacterial culture) by HPLC [21].

Assay for HCN

HCN production test was done as demonstrated earlier [22]. The bacterial isolates were grown at 28°C on a rotary shaker in tryptic soy broth. Whatman No. 1 filter paper was cut into uniform strips of 9 cm long and 0.5 cm wide, saturated with alkaline picrate solution and placed inside the conical flasks in a hanging position. After incubation at 28°C for 48 h, the sodium picrate present in the filter paper was observed for a change in color, and absorbance was measured at 625 nm with five replications.

Assay for Chitinase

Colloidal chitin was prepared as described earlier by Berger and Reynolds [23]. The fluorescent pseudomonads isolates were grown at 28°C on rotary shaker in 250 ml conical flasks with 100 ml of chitin-peptone medium (colloidal chitin 0.2%, glucose 0.5%, peptone 0.2%, K_2HPO_4 0.1%, $MgSO_4$ $7H_2O$ 0.05% and NaCl 0.05%, pH 6.8) [24]. After 96 h of incubation, the cultures were centrifuged at 12,000×*g* for 15 min at 4°C, and the supernatant was used as enzyme source. The absorbance value was read at 280 nm using a spectrophotometer. *N*-acetylglucosamine (GlcNac) was used as a standard and the enzyme activity was expressed as nmol GlcNac/min/ml.

Genetic Diversity of Fluorescent Pseudomonads

Extraction of Total Genomic DNA

Genomic DNA of the fluorescent pseudomonads was extracted by GenElute Bacterial Genomic DNA Kit (Sigma, USA). Amplification of 16S rDNA region was done using bacterial universal primers pA (5′-AGAGTTTGATCCTGG CTAG-3′) and rP2 (5′-ACGGCTACCTTGTTACGACT T-3′) as described earlier [25].

PCR-RFLP Analysis of 16S rDNA

16S rDNA-PCR products were purified by QIA quick PCR purification kit (Qiagen), 100-ng purified PCR products were digested with three units of restriction endonucleases *Hae*III, *Alu*I, and *Msp*I (Bangalore GeNei, India) as per instructions of the manufacturer. The digested products together with marker (100 bp, Bangalore GeNei, India) were resolved by gel electrophoresis (60 V cm^{-1}) on 2.5% agarose gels in 1× TAE buffer containing 10 µg ml^{-1} ethidium bromide (EB). Gels were photographed by gel documentation system (Syngene, Cambridge, UK).

Detection of Known Antibiotic Coding Genes

The two antibiotic coding genes, viz., 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin (PLT) were detected as described earlier [6]. For the amplification of DAPG (745 bp), the two primers, viz., Phl2a (5′-GAGGACGTC GAAGACCACCA-3′) and Phl2b (5′-ACC GCAGCATC GTGTATGAG-3′) were used; PLT (779 bp) region was amplified by the primers, i.e., PltBf (5′-CGGAGCAT GGACCCCCAGC-3′) and PltBr (5′-GTGCCCGATATTG GTCTTGACCGAG-3′).

Table 2 Biochemical properties of 25 antagonistic potential fluorescent pseudomonads

Fluorescent pseudomonads isolates	Gram reaction	Shape	Nitrate reduction	Catalase	Oxidase	Methyl red	Starch hydrolysis	Lipase	Fluorescent pigments	Motility	Glucose utilization
PfPMG	−	Rod	+	+	+	−	−	+	+	+	+
Pf146	−	Rod	−	+	+	+	+	−	+	+	+
Pf140	−	Rod	+	+	+	−	−	+	+	+	+
Pf351	−	Rod	+	+	+	−	−	+	+	+	+
Pf125	−	Rod	+	+	+	+	−	+	+	+	+
Pf165	−	Rod	−	+	+	−	−	+	+	+	+
Pf214	−	Rod	+	+	+	−	+	+	+	+	+
Pf87	−	Rod	−	+	+	−	−	+	+	+	+
Pf355	−	Rod	+	+	+	−	+	−	+	+	+
Pf245	−	Rod	−	+	+	−	−	+	+	+	+
PfM11	−	Rod	+	+	+	+	−	+	+	+	+
Pf12	−	Rod	+	+	+	−	+	−	+	+	+
Pf13	−	Rod	−	+	+	−	−	+	+	+	+
Pf14	−	Rod	+	+	+	−	−	+	+	+	+
PfM31	−	Rod	+	+	+	−	−	+	+	+	+
Pf197	−	Rod	+	+	+	−	−	+	+	+	+
Pf373	−	Rod	−	+	+	+	+	−	+	+	+
Pf264	−	Rod	−	+	+	+	−	−	+	+	+
Pf238	−	Rod	+	+	+	−	−	+	+	+	+
Pf213	−	Rod	+	+	+	−	−	−	+	+	+
Pf83	−	Rod	−	+	+	−	−	+	+	+	+
Pf130	−	Rod	−	+	+	−	−	+	+	+	+
Pf141	−	Rod	+	+	+	−	+	−	+	+	+
PfM3	−	Rod	−	+	+	−	−	+	+	+	+
Pf59	−	Rod	−	+	+	−	−	+	+	+	+
2099	−	Rod	+	+	+	−	−	+	+	+	+
2582	−	Rod	+	+	+	−	−	+	+		+
2026	−	Rod	+	ND	ND	ND	−	+	+	+	+
673	−	Rod	−	ND	−	ND	−	−	+	+	+

'+' positive for the test; '−' negative for the test; 'ND' not determined whether positive or negative for the test; from PfPMG to Pf59 are fluorescent pseudomonads isolated from different tea gardens; 2099, *Pseudomonas fluorescens* NICM 2099^T; 2582, *P. aeruginosa* MTCC 2582^T; 2026, *P. aureofaciens* NICM 2026^T; 673, *P. syringae* MTCC 673^T

Data Analysis

The significant differences were determined by analysis of variance (ANOVA) by Students' t test. The significance level for all the analyses was $P = 0.05$.

For the analysis of 16S rDNA-RFLP data, the character state '1' was given for a band, which could be clearly and reproducibly detected in the gel and '0' was assigned if it was absent, or if it was not possible to determine. The data matrix thus generated was calculated by Jaccard's [26] similarity coefficient for each pairwise comparison. Dendogram was constructed from the similarity matrix by the unweighted pair group method with arithmetic mean (UPGMA) [27].

Results

Morphological and Biochemical Characterization

Among the bacterial isolates, 25 were confirmed as fluorescent pseudomonads based on morphological and biochemical tests such as gram reaction, nitrate reduction, catalase, oxidase, methyl red, starch hydrolysis, lipase production, fluorescent pigments, and sugar (glucose) utilization test (Table 2). Microscopic observation showed that all the 25 isolates were rod shaped, motile, gram-negative, and fluorescent in the presence of UV light. All the isolates were positive in catalase, oxidase, and glucose utilization test. However, in other biochemical tests, the

Table 3 Antimicrobial activity of bioactive metabolite of fluorescent pseudomonads extracted in 10% dimethyl sulfoxide

Sl. No.	Fluorescent pseudomonads isolates	*Fusarium oxysporum* f. sp. *raphani*	*Fusarium oxysporum* f. sp. *ciceri*	*Fusarium semitectum*	*Rhizoctonia solani*	*Staphylococcus aureus*	*Escherichia coli*	*Klebsiella pneumoniae*
1	PfPMG	+++	+++	+++	+++	+++	–	–
2	Pf146	++	–	+++	+++	+++	–	+
3	Pf140	+++	–	+++	–	+	–	++
4	Pf351	+++	+++	+++	+++	+++	–	–
5	Pf125	+++	+++	+++	+++	+++		
6	Pf165	+++	–	+++	+++	+++	++	++
7	Pf214	–	–	+++	+++	+	++	+
8	Pf87	+++	+++	+++	+++	+++	+	+
9	Pf355	+++	+++	+++	+++	+++	+	+
10	Pf245	+++	+++	+++	+++	+++	+	+
11	PfM11	+++	+++	+++	+++	+++	–	–
12	Pf12	+++	–	+++	+++	+++	–	–
13	Pf13	+++	+++	+++	+++	+++	++	+
14	Pf14	+++	+++	+++	+++	+++	++	++
15	PfM31	–	–	+++	+++	+++	+	+
16	Pf197	+++	+++	+++	–	+	–	–
17	Pf373	+++	+++	+++	+++	+++	–	–
18	Pf264	–	+++	+++	+++	+++	–	–
19	Pf238	–	+++	+++	+++	+++	–	–
20	Pf213	+++	+++	+++	–	+	–	–
21	Pf83	+++	+++	–	+++	+++	+	+
22	Pf130	+++	+++	+++	+++	+++	+	–
23	Pf141	–	–	+++	–	+	+	–
24	PfM3	+++	+++	+++	+++	+++	–	–
25	Pf59	+++	+++	+++	+++	+++	++	++

'–' no inhibition; '+' low inhibition (1–10 mm); '++' medium inhibition (11–20 mm); '+++' strong inhibition (21 mm or above)

isolates showed a variation, e.g., out of 25 isolates, 14 isolates (PfPMG, Pf140, Pf351, Pf125, Pf214, Pf355, PfM11, Pf12, Pf14, PfM31, Pf197, Pf238, Pf213, and Pf141) positive in nitrate reduction test; five isolates, viz., Pf146, Pf125, PfM11, Pf373, and Pf264 positive in methyl red test; however, six isolates (Pf146, Pf214, Pf355, Pf12, Pf373, and Pf141) and 18 isolates (PfPMG, Pf140, Pf351, Pf125, Pf165, Pf214, Pf87, Pf245, PfM11, Pf13, Pf14, PfM31, Pf197, Pf238, Pf83, Pf130, PfM3, and Pf59) showed starch hydrolysis and lipase activity, respectively (Table 2).

Antimicrobial Activity

To determine the antagonistic activity of the secondary metabolite produced by these isolates, they were extracted and tested against the pathogens such as *F. oxysporum* f. sp. *raphani, F. oxysporum* f. sp. *ciceri, F. semitectum*, and *R. solani* as well as bacterial pathogens such as *S. aureus, E. coli*, and *K. pneumoniae.* Among these isolates, PfPMG, Pf351, Pf125, Pf87, Pf355, Pf245, PfM11, Pf13, Pf14, Pf373, Pf130, PfM3, and Pf59 showed strong antifungal activity against all the fungal pathogens tested (Table 3). However, other isolates showed mixed reactions in antagonistic activity. For example, Pf83 exhibited strong antifungal activity against the fungal pathogens (*F. oxysporum* f. sp. *raphani, F. oxysporum* f. sp. *ciceri*, and *R. solani*) except *F. semitectum*. Similarly, Pf197 and Pf213 recorded strong antifungal activity against *F. oxysporum* f. sp. *raphani, F. oxysporum* f. sp. *ciceri*, and *F. semitectum* but not against *R. solani*. Pf140 strongly inhibited the growth of *F. oxysporum* f. sp. *raphani*, and *F. semitectum*, but could not inhibit *F. oxysporum* f. sp. *ciceri* and *R. solani.* Most of the fluorescent pseudomonads isolates strongly inhibited the growth of gram-positive bacterium *S. aureus*; moreover, some isolates viz., Pf146, Pf140, Pf165, Pf214, Pf87, Pf355, Pf13, Pf14, Pf83, Pf130, Pf141, and Pf59 also inhibited the gram-negative bacteria, *E. coli* and *K. pneumoniae* (Table 3).

Antimicrobial Traits

All the isolates variably produced siderophore, salicylic acid, and HCN. The maximum siderophore production was recorded with the isolate Pf59 followed by Pf14, Pf13, Pf197, and PfM3 (Fig. 1a); however, the lowest production was recorded with Pf355. Likewise, the SA production was recorded to be the maximum in Pf59 followed by Pf197, Pf13, Pf264, and PfM31 (Fig. 1b). Similarly, the isolate Pf59 showed the maximum production of HCN, followed by Pf197, Pf83, Pf125, and Pf355; the lowest activity was recorded in Pf12 (Fig. 1c). A significant relationship between antagonistic potential of the fluorescent isolates and their level of production of siderophore and SA was observed.

Out of 25 isolates, only 13 isolates, viz., Pf59, Pf197, Pf245, Pf355, Pf351, PfM3, Pf83, Pf14, Pf87, PfPMG, Pf140, Pf214, and PfM31 produced cell wall-degrading enzyme, chitinase. Among the isolates, Pf59 exhibited the highest chitinase activity followed by Pf197, Pf245, Pf355, Pf351, and then other isolates (Fig. 1d).

PCR-RFLP Analysis

The 16S rDNA of all the 24 isolates and reference strains were amplified with the primers pA and rP2. Gel electrophoresis of the undigested PCR products revealed that all the isolates produced a single band of about 1440 bp (Fig. 2). The RFLP analysis showed huge variations among the isolates. The restriction patterns obtained after digestion of the amplified 16S rDNA fragment with *Hae*III revealed 16 restriction patterns in the ranges of 100–1000 bp (Fig. 3a). Digestion with *Hae*III revealed a common banding pattern at 180 bp for all other isolates except Pf351, Pf214, Pf83, Pf141, and PfM3. Similarly, except the isolates Pf83, Pf141, PfM3, and the reference strain, *P. aeruginosa* MTCC 2582, a band at 220 bp was observed in all other isolates. The fragments at 240 bp and 500 bp were found only with Pf141 and PfM3 isolates. A common band at 100 bp was observed with the isolates Pf165, Pf87, Pf355, Pf245, PfM11, Pf12, Pf13, Pf141, *P. syringae* 673, and *P. fluorescens* 2099. The isolates Pf165, Pf355, and the reference strain, *P. syringae* 673, have shown a common band at 900 bp.

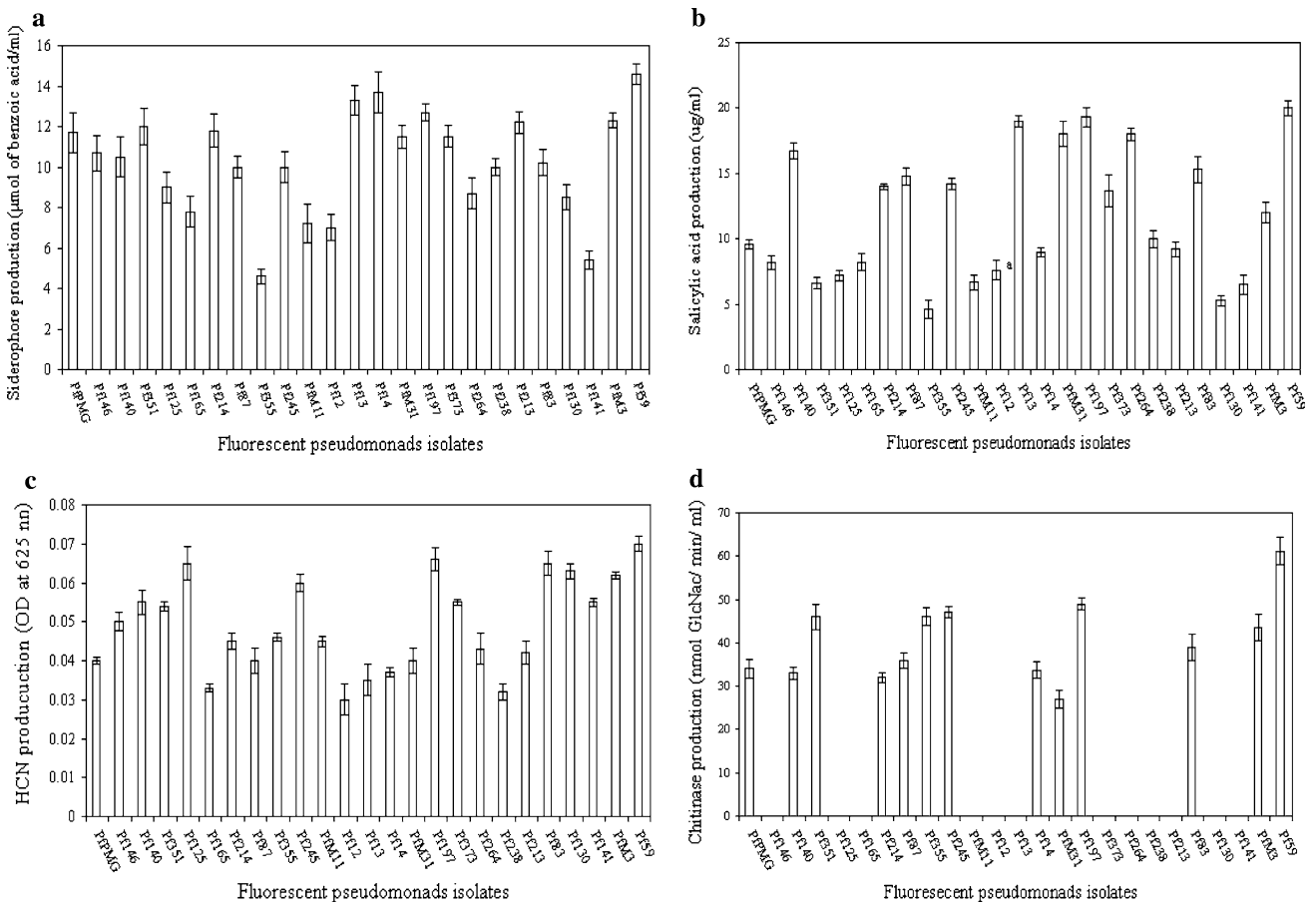

Fig. 1 Antimicrobial traits of fluorescent pseudomonads isolates: **a** siderophore production; **b** salicylic acid production; **c** HCN production, and **d** chitinase production

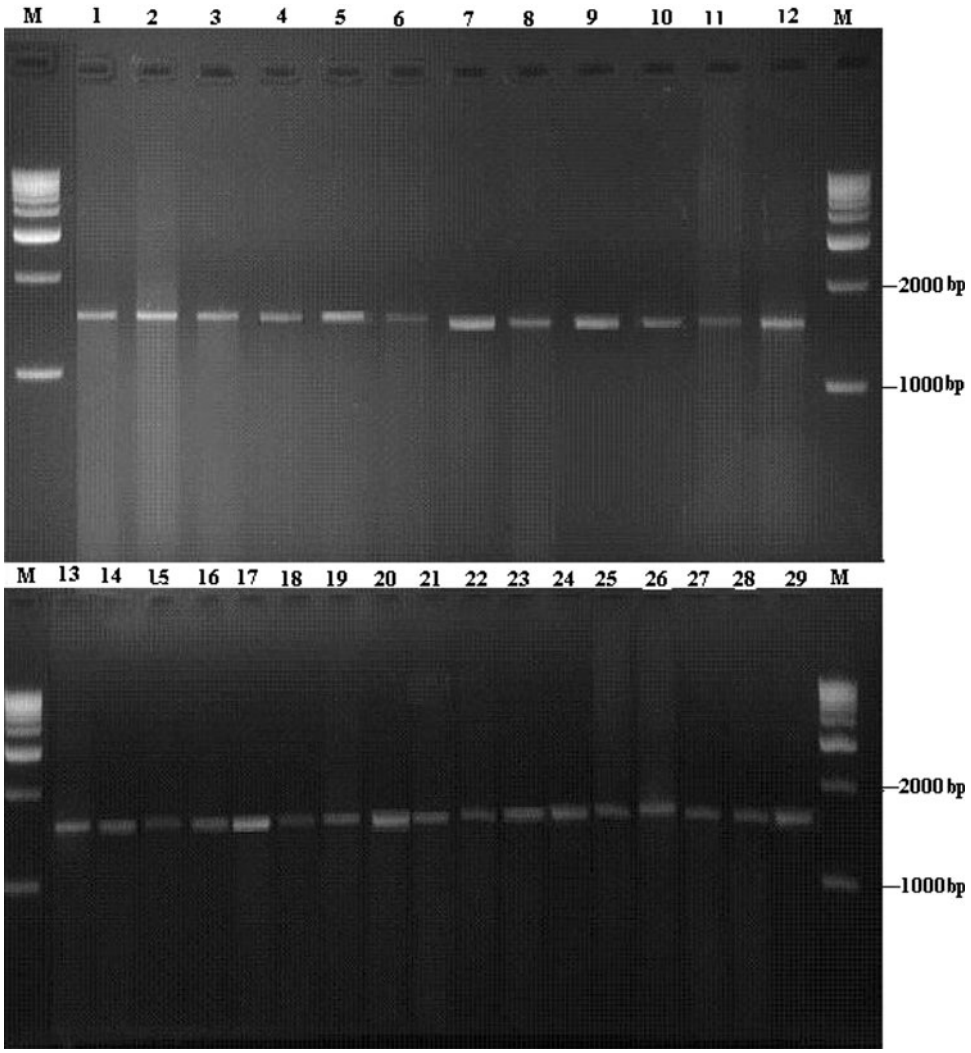

Fig. 2 Agarose gel electrophoresis of PCR amplified 16S rDNA; Lane name with respective isolates: M-1 kb molecular marker; 1-PfPMG; 2-Pf146; 3-Pf140; 4-Pf351; 5-Pf125; 6-Pf165; 7-Pf214; 8-Pf87; 9-Pf355; 10-Pf245; 11-PfM11; 12-Pf12; 13-Pf13; 14-Pf14; 15-PfM31; 16-Pf197; 17-Pf373; 18-Pf264; 19-Pf238; 20-Pf213; 21-Pf83; 22-Pf130; 23-Pf141; 24-PfM3; 25-Pf59; 26-*Pseudomonas aeruginosa* MTCC 2582^{T}; 27-*P. syringae* MTCC673^{T}; 28-*P. fluorescens* NICM 2099^{T}; 29-*P. aureofaciens* NICM 2026^{T}

Digestion of 16S rDNA product with *Alu*I gave 13 restriction patterns (Fig. 3b). Except the isolates PfPMG, Pf351, Pf83, Pf141, PfM3, and Pf59, the fragment at 180 bp was common to all other isolates. Similarly, all other isolates except the isolate Pf83 have a common banding pattern at about 200 bp. The restriction fragment at 400 bp was also common to all other isolates except PfPMG, Pf83, Pf141, PfM3, and *P. fluorescens* 2099. A pair of unique bands at 750 bp and 150 bp was observed corresponding to the isolates Pf355 and Pf59, respectively.

*Msp*I restriction endonucleases produced 20 nos. of restriction patterns (data not shown). Common banding pattern at 600 and 150 bp were found for all the isolates; however, the isolate Pf245 possessed a banding pattern at 400 bp.

Dendrogram was derived from the distance matrix by the UPGMA, and a total of 25 different combinations of restriction patterns were recorded among 29 isolates (including four reference strains) (Fig. 4). A critical analysis of the results shows a major division into two clusters (A and B) at 72% similarity coefficient value (Fig. 4). The cluster A comprised only two isolates, PfM3 and Pf141 which were about 80% similar whereas cluster B has 27 isolates and also the reference strains. However, the cluster B further divided into two sub-clusters (C and D) at 74% similarity coefficient value where isolate Pf59 was separated along. Then, the sub-cluster D was again divided into two sub-clusters, i.e., E and F at 75% similarity coefficient value where E contained only the isolate Pf355. The sub-cluster F contained 25 isolates which were further separated into two sub-clusters with (approx.) 82% similarity. The isolates Pf13, Pf14, Pf165, and Pf197 showed 100% similarity. Similarly, the isolate Pf373 was 100% identical with the isolate Pf264 which were isolated from the same tea garden (Chenijan Tea Garden). The isolates PfPMG, Pf146, Pf140, and Pf351 were with 87% similarity to the reference strain *P. aeruginosa* MTCC2582. The isolate Pf245 was 94% similar with the reference strain *P. syringae* MTCC673 which has (approx.) 87% similarity with the isolates Pf125, PfM11, Pf12, Pf13, Pf14, Pf165, Pf197,

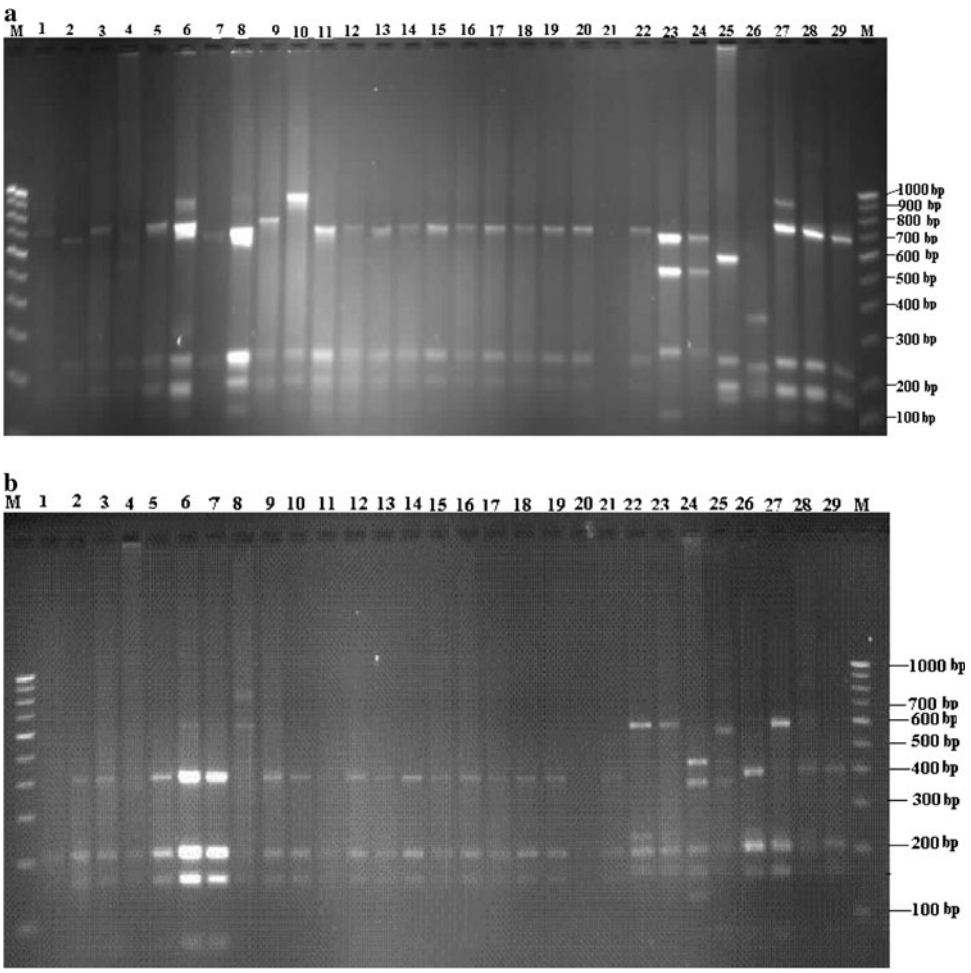

Fig. 3 **a**, **b** Restriction patterns of PCR amplified fragment of 16S rDNA digested with *Hae*III and *Alu*I. Lane name with respective isolates: **a** M-100 bp molecular marker; 1-PfPMG; 2-Pf146; 3-Pf140; 4-Pf351; 5-Pf125; 6-Pf165; 7-Pf214; 8-Pf87; 9-Pf355; 10-Pf245; 11-PfM11; 12-Pf12; 13-Pf13; 14-Pf14; 15-PfM31; 16-Pf197; 17-Pf373; 18-Pf264; 19-Pf238; 20-Pf213; 21-Pf83; 22-Pf130; 23-Pf141; 24-PfM3; 25-Pf59; 26-*Pseudomonas aeruginosa* MTCC 2582^T; 27-*P. syringae* MTCC 673^T; 28-*P. fluorescens* NICM 2099^T; 29-*P. aureofaciens* NICM 2026^T. **b** M-100 bp molecular marker; 1-PfPMG; 2-Pf146; 3-Pf140; 4-Pf351; 5-Pf165; 6-Pf214; 7-Pf87; 8-Pf355; 9-Pf245; 10-PfM11; 11-Pf12; 12-Pf13; 13-Pf14; 14-PfM31; 15-Pf197; 16-Pf373; 17-Pf264; 18-Pf238; 19-Pf213; 20-Pf83; 21-Pf130; 22-Pf141; 23-PfM3; 24-Pf59; 25-*Pseudomonas aeruginosa* MTCC 2582^T; 26-*P. syringae* 673^T; 27-*P. fluorescens* NICM2099^T; 28-*P.aureofaciens* 2026^T; 29-Pf125

PfM31, Pf373, Pf264, Pf238, Pf213, Pf130, Pf83, and Pf214. The isolate Pf87 has recorded about 84% similarity with *P. aureofaciens* NICM2026 (Fig. 4).

Detection of Known Antibiotic Coding Genes

Genomic DNA samples of the fluorescent pseudomonads isolates were tested as templates using gene-specific primers. Isolate Pf12 was amplified to revel the DNA fragment of 745 bp for DAPG and isolate Pf373 was amplified DNA fragment of 779 bp for PLT (Fig. 5).

Discussion

The tea root–soil interface in the tropical rain forest consists of a diverse community of microbes which plays a central role in gross production and nutrient cycling. In this study, we analyzed the genotypic and functional diversity of fluorescent pseudomonads and its possible consequences in terms of antagonistic activity. Antagonistic activities of many strains of fluorescent pseudomonads against different pathogens have been reported which are normally influenced by the production of several secondary metabolites like 2,4-diacetylphloroglucinol (2,4-DAPG), PLT, HCN, phenazine, siderophore, secretion of cell wall-degrading enzymes, and biosurfactants [4, 28, 29].

Although role of siderophores in disease suppression by fluorescent pseudomonads is controversial [30], Kloepper et al. [1] observed that *P. putida* strain B10 suppressed *Fusarium* wilt and take-all, but this suppression was lost when the soil was amended with iron, which repressed siderophore production in this strain. Our observation also indicated a correlation between growth suppression of the pathogens and production of siderophore by the isolates of fluorescent pseudomonads. Salicylic acid is a phenolic compound that affects a variety of biochemical and molecular events associated with induction of disease resistance. SA has been shown to play an important role in expression of both local resistance controlled by major genes and systemic induced resistance developed after an initial pathogen attack [31]. Our results indicated significant relationship between antagonistic potential of some fluorescent pseudomonads isolates and their level of production of SA.

Among the antibiotic substances involved in the biocontrol of phytopathogens, HCN is a toxic secondary

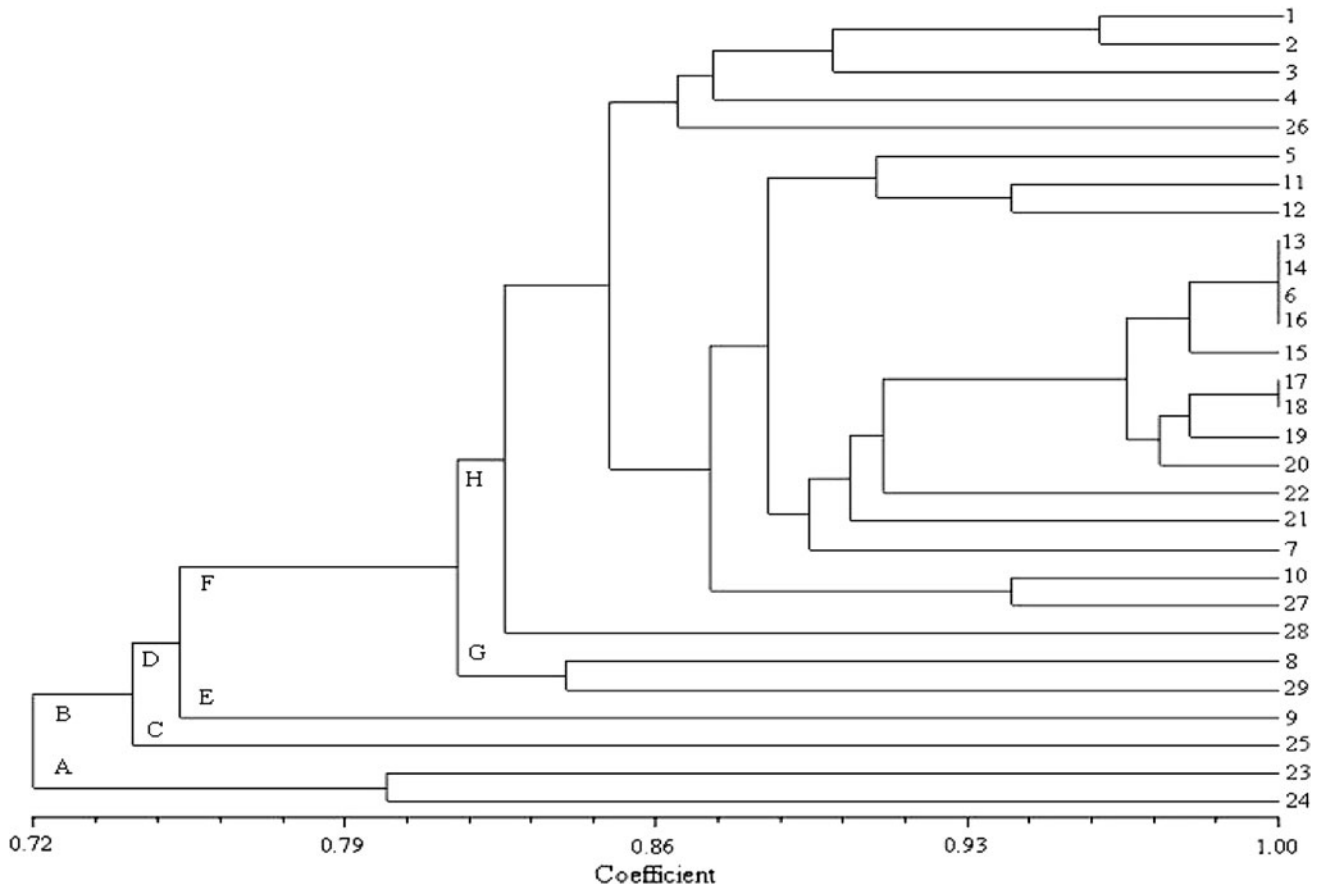

Fig. 4 Combined dendrogram of antagonistic potential fluorescent pseudomonads isolates based on cluster analysis of 16S rDNA-RFLP products with restriction endonuclease *Hae*III, *Alu*I and *Msp*I using the UPGMA algorithm and the Jaccard coefficient; 1-PfPMG; 2-Pf146; 3-Pf140; 4-Pf351; 5-Pf125; 6-Pf165; 7-Pf214; 8-Pf87; 9-Pf355; 10-Pf245; 11-PfM11; 12-Pf12; 13-Pf13; 14-Pf14; 15-PfM31; 16-Pf197; 17-Pf373; 18-Pf264; 19-Pf238; 20-Pf213; 21-Pf83; 22-Pf130; 23-Pf141; 24-PfM3; 25-Pf59; 26-*Pseudomonas aeruginosa* MTCC 2582^T; 27-*P. syringae* MTCC 673^T; 28-*P. fluorescens* NICM 2099^T; 29-*P. aureofaciens* NICM 2026^T

metabolite that is produced by many rhizosphere microorganisms, especially by fluorescent pseudomonads [32]. The role of antibiotic HCN produced by fluorescent pseudomonads in the suppression of different diseases has been documented [28]. In this study, the fact that the highly antagonistic activity-mediated isolates produced more HCN might have contributed to the inhibition of growth of the pathogens. Production of lytic enzyme, chitinase, and β-1,3-glucanase by several PGPR strains is considered as a major antagonistic property of these strains [7, 33]. These lytic enzymes have hydrolytic action and degrade fungal cell wall of many pathogenic fungi [33]. The results of this study indicate that there was a significant relationship between antagonistic activity of the some isolates (i.e. PfPMG, Pf140, Pf351, Pf214, Pf87, Pf355, Pf245, Pf14, PfM31, Pf197, Pf83, PfM3, and Pf59) and their chitinase-producing capacity, demonstrating that production of the enzyme may be the cause of antagonistic activity of the fluorescent pseudomonads against the fungal pathogens. However, some of the isolates did not show chitinase activity and still showed antagonistic activity against the pathogens, therefore, the antagonistic activity of these isolates may not be correlated to the capacity of these isolates to chitinase production.

Productions of DAPG and PLT have been shown to be important mechanisms of biological control of a wide variety of plant pathogens by fluorescent *Pseudomonas* spp. [29]. However, in this study, out of 25 isolates, only two, viz., Pf12 and Pf373, exhibited the presence of known antibiotic genes DAPG and PLT, respectively; others did not show the known antibiotic genes. Therefore, the antagonistic activity of these isolates may be attributed to some other factors rather than the known antibiotics.

In this study, a combination of three tetra cutter restriction endonuclease *Hae*III, *Alu*I, and *Msp*I permitted a resolution level as comparable to the earlier reports [34]. In 16S rDNA-RFLP with *Hae*III and *Alu*I, there was a significant difference observed in all the 25 fluorescent pseudomonads isolates that belonged to the tea rhizosphere (Fig. 4). In this study, it has been observed that some

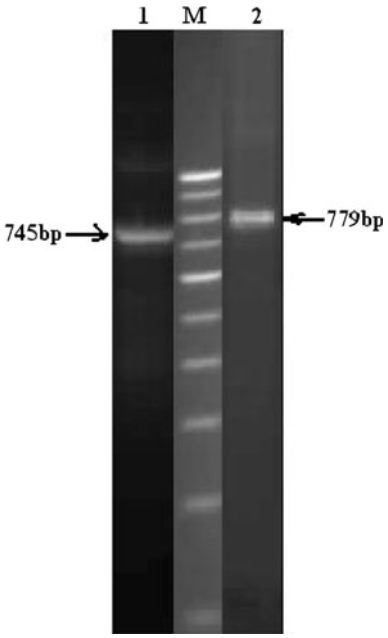

Fig. 5 PCR amplification of antibiotic coding regions: 1. 2,4-diacetylphloroglucinol (DAPG) in Pf12; M. 100 bp molecular marker; 2. pyoluteorin (PLT) in Pf373

isolates of different tea garden (even tea gardens of different district also) belong to the same cluster; similarly, some isolates of same district (even same tea garden) have also set off to other clusters. The genetic variation of these isolates might be due to mutation [7, 35] and other genetic changes like recombination [36]. Mutation rates in bacteria are known to generally increase under stress because of the SOS response and reduced ability to deal with DNA-damaging free radicals generated by metabolism [37].

Overall the findings of this study suggest that (i) fluorescent pseudomonads isolated from rhizospheric soil of tea have shown strong antimicrobial properties against the fungal pathogens and gram-positive bacteria—subsequently, more than one mechanism may be involved in the suppression of the pathogens, and (ii) high genetic diversity was observed among the isolates.

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