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Genetic Diversity Among Cold-Tolerant Fluorescent *Pseudomonas* Isolates from Indian Himalayas and Their Characterization for Biocontrol and Plant Growth-Promoting Activities

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Abstract In Uttarakhand, the Organic State of India, where soils in most farming situations are deficient in nutrients and loss of crops due to soil- and seed-borne pathogens is rampant, use of native plant growth-promoting rhizobacteria (PGPRs) possessing biocontrol (BC) activities holds promise. In view of this, 600 native cold-tolerant rhizospheric bacterial isolates were collected from Uttarakhand Himalayas, of which 336 were confirmed as fluorescent *Pseudomonas* spp. On the basis of specific biochemical tests, these were characterized into three major groups: *P. fluorescens* (308 isolates), *P. aeruginosa* (20 isolates), and *P. putida* (8 isolates). Most of the isolates could grow at 8°C after 12 h of incubation, confirming their cold tolerance. In vitro biocontrol assays revealed that of 336 isolates, 74 were antagonistic to *Rhizoctonia solani* and 91 to *Fusarium solani*, the two major pathogens associated with root-rot complex in vegetables widespread in the region. Simultaneously, good HCN producers (33 isolates), siderophore producers (80 isolates), and P solubilizers (49 isolates) were also identified, which could increase the biocontrol and plant growth-promoting efficacies of the putative PGPRs. Among the different species and biovars, *P. fluorescens* biovar-I had the maximum number of potential isolates with BC and plant growth-promoting (PGP) activities. In French bean, under polyhouse and field conditions, five isolates (Pf-173, Pf-193, Pf-547, Pf-551, and Pf-572) showed good BC and PGP activities as up to 93% reduction in root rot was achieved. A combination of all five isolates was found to be best with respect to BC and PGP activities. In a set of 59 fluorescent *Pseudomonas* isolates, RAPD-PCR analysis, using three random oligodecamer primers, revealed high diversity and formed ten distinct clusters, corresponding to the host of origin (annual or perennial) or habitat (farming situations) of the isolates. The amount of diversity revealed in the set of fluorescent *Pseudomonas* isolates could represent enormous diversity that exists in the wild that could be exploited for improved BC and PGP activities of the PGPRs. For the first time, this study led to a large-scale characterization and repositioning of fluorescent pseudomonads from the Indian Himalayas.

Keywords Biocontrol · Cold-tolerant · Genetic diversity · Plant growth promotion · *Pseudomonas fluorescens*

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Introduction

Research on plant growth-promoting rhizobacteria (PGPR) over the last three decades has unraveled their efficacy in improving plant growth by increasing seed emergence, plant height, weight, and ultimately crop yield (Kloepper and others 1980, 1986). Among the most common PGPRs are *Acinetobacter*, *Azotobacter*, *Bacillus* spp., fluorescent *Pseudomonas* spp., *Rhizobium* spp. However, during the last decade much of the research has been focused on fluorescent pseudomonads because of their broad-spectrum activities for plant growth promotion and biocontrol of different plant diseases. Diverse populations of *Pseudomonas* spp. have been reported to play a major role in plant

growth promotion and suppression of root diseases and are the subjects of ongoing investigations worldwide (Keel and others 1996; McSpadden Gardener and others 2000; Picard and others 2000). In general, diverse populations of PGPR provide better resources for the improvement of plant growth promotion and biocontrol ability, as different strains possess varied modes of action and survival in diverse environmental conditions (Stutz and others 1986; Weller 1988; Ramesh Kumar and others 2002).

In view of the above, we focused our long-term objective on establishing a repository of cold-tolerant fluorescent pseudomonads possessing biocontrol (BC) and plant growth-promoting (PGP) activities. Putative candidates possessing desirable BC and PGP activities would be introduced to farmers' fields where chronic recurrent cycles of diseases take a heavy toll on crops each season and lead to severe losses for small and marginal farmers. Such losses are characteristic of hill farming as soils in most farming situations in the Uttarakhand Himalayas are acidic and poor in organic matter and available phosphorus.

To counter crop losses, farmers resort to indiscriminate and often irrelevant crop protection measures which, over time, have led to serious situations of resurgence of pest populations, increased crop losses, and environmental and ground water pollution. Resistance development in pest populations might also be involved. Use of synthetic chemicals for the management of diseases is largely uneconomical and does not fit within the framework of "organic farming," which is the State policy. Low-volume, high-value off-season vegetable cultivation is more popular among small farmers and is the major source of their livelihood. Unfortunately, like other agriculture crops, cultivation of vegetables can be threatened by different deadly pathogens. Among these, *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (Jones) Snyd. & Hans and *Rhizoctonia solani* are major threats to successful cultivation of vegetable crops and cause damping off and root rot in different vegetables. In the case of French bean, *R. solani* is more vicious and also causes collar rot, web blight, and aerial blight, which are equally destructive to the crop. Low soil fertility and acidic soils favor disease severity (Hagedorn 1984). The off-season vegetable crops are cultivated mostly at higher altitudes (1000–2100 masl), where soil temperatures range from 6 to 18°C during different cropping seasons. Therefore, availability of native cold-tolerant PGPRs is imperative for candidate PGPRs for BC and PGP activities.

In the present study, a collection of rhizospheric bacteria of different annuals and perennials was obtained from farmers' fields at an altitude ranging from 1700 to 2100 masl. The bacteria were subjected to biochemical and molecular characterization. In addition, five putative isolates were selected on the basis of their in vitro antagonism and PGP activities and were evaluated for BC and PGP activities in polyhouse and field conditions in French bean, which is one of the important off-season vegetables grown in this region. To the best of our knowledge, this study is the first large-scale characterization of cold-tolerant fluorescent pseudomonads from the Indian Himalayas.

Materials and Methods

Isolation of Bacterial Cultures

Native isolates were obtained from the rhizospheric soils of annuals and perennials from different farming situations in the Garhwal division of Uttarakhand Himalayas. Isolation of bacteria from rhizospheric soil samples was carried out on King's medium B (King and others 1954) plates on the basis of characteristic yellow-green fluorescence under UV light. Mother cultures were stored in half-strength King's broth at −20°C.

Fungal Pathogens

Two potential fungal pathogens (*Rhizoctonia solani* and *Fusarium solani* f. sp. *pisi*), involved in root-rot complex in vegetables, were obtained from the well-characterized culture stock being maintained in the Plant Pathology Laboratory, Hill Campus, Ranichauri.

Biochemical Characterization of Bacterial Isolates

Biochemical tests specific for *Pseudomonas* sp., such as gelatin hydrolysis (Sadowsky and others 1983), the oxidase test (Kovaks 1956), catalase (Graham and Parker 1964), levan formation (Lelliott and others 1966), arginine dihydrolysis (Lelliott and others 1966), starch hydrolysis (Lelliott and others 1966), and ammonification (Lee and Kobyashi 1989), were performed to distinguish fluorescent pseudomonads from rhizospheric populations of other bacteria. Biochemical tests were performed as per standard methods and the results were authenticated by referring to *Bergey's manual of determinative bacteriology* (Holt and others 1994).

Growth at Different Temperatures

Soil temperature at different farming situations varied from 6 to 18°C during different cropping seasons. Therefore, all the fluorescent pseudomonads (fPs) were evaluated for their ability to grow at a range of temperatures. Isolates were streaked on KB plates and incubated at 4, 6, 8, 10, 12, 15, 20, 25, and 30°C. Plates were observed for bacterial growth at different time intervals (6, 12, 18, 24, 36 h).

Screening of Fluorescent *Pseudomonas* Isolates for In Vitro Antagonism

In vitro characterization of fPs was carried out against both phytopathogenic fungi, *Rhizoctonia solani* and *Fusarium solani*, on King's medium B. The fungi were grown and maintained on PDA. Four bacterial isolates were checked simultaneously on a plate for their antagonistic activity against the target pathogens. Antagonistic efficacy of isolates was measured on the basis of zone of inhibition produced against the fungal growth.

To understand the mode of inhibition of fungal growth by *Pseudomonas* isolates, a disk of fungal growth from the leading edge in the plates was picked and placed on a fresh KB plate and incubated at 25 ± 2°C for 1 week. Thereafter, plates were observed for the growth of fungi. Also, a microscopic study of affected fungal mycelium in a dual-culture plate was carried out to confirm the mode of inhibition. For this, mycelium was sampled from the edge of fungal growth using a sterilized inoculation needle and mounted on slides using lactophenol. Slides were then observed for lysis or mycelial deformity under bright light in an Olympus Research Vanox-S microscope.

Antagonistic efficacy of *Pseudomonas* isolates was calculated using the formula: $[(A - B)/A] \times 100$, where A is the diameter of fungal growth in the control plate and B is the diameter of fungal growth up to the zone of inhibition in the test plate. Only those fP isolates that produced a good zone of inhibition (>0.4 cm) were considered potential antagonists. The antagonistic fP isolates were assigned to one of three grades: A (inhibition zone >0.8 cm), B (0.4–0.7 cm), and C (<0.4 cm). Fluorescent *Pseudomonas* isolates with a C grade were not considered potential antagonists.

Evaluation for HCN Production

A modified method of Miller and Higgins (1970) was used to evaluate all the isolates for HCN production using tryptic soy agar plates. Lids of the plates were lined with filter paper dipped in alkaline picric acid solution and the plates were incubated at 28 ± 2°C for 3–5 days. After incubation, plates were observed for a change in color of the filter paper from yellow to orange, brown, or brick red. Development of orange, brown, or brick red color indicated HCN production (positive reaction).

Evaluation for Phosphate Solubilization

The ability of the pseudomonads to solubilize phosphate was assessed on Pikovaskya's agar medium in two steps. First, six to ten *Pseudomonas* isolates (on one medium plate) were spot inoculated in triplicate and incubated at 28 ± 2°C for 72 h. After incubation, plates were observed for development of a phosphorus solubilization zone around the bacterial growth. Isolates found positive for P solubilization were reconfirmed in the second step, wherein isolates were round streaked on the medium plates and incubated under the same conditions as above. After incubation, plates were observed for a solubilization zone. Phosphate solubilization efficacy (PSE) of the fluorescent pseudomonads was estimated by the formula: (diameter of solubilization zone/diameter of bacterial growth) × 100.

Evaluation for Siderophore Production

The ability of the isolates to produce siderophores was assessed on "Chrome azurol S" agar medium following the method of Mayer and Abdullah (1978). This ability was again evaluated in two steps as described for P solubilization. After incubation at 28 ± 2°C for 72 h, the plates were observed for an orange halo around the bacterial growth. Percent siderophore-producing efficacy (SPE) of the pseudomonads was estimated by the formula: (diameter of orange halo/diameter of bacterial growth) × 100.

Evaluation for Biocontrol and Plant Growth-Promoting Activities Under Controlled Conditions

BC and PGP activities of fPs were evaluated in French bean (var. *Pusa contendor*) in an environmentally controlled screen house. *Rhizoctonia solani* was used as the target pathogen. Two sets of experiments [17 treatments (Table 1), each with three replications] were maintained. In experiment I, unsterilized soil provided natural soil conditions for the *Pseudomonas fluorescens* isolates (Pfs), and in experiment II, 250 mg of pathogen inoculum was added to the top 1 kg of soil in each pot. In both experiments seeds were treated (6 g kg^{-1} seed) with the talc formulation of Pfs. Ten seeds per pot were sown at a depth of 1 cm. Soils were moistened once a day. Germination was recorded every day after the first seed germinated and continued until full germination was achieved. Similarly, mortality was recorded daily. After 30 days all plants were uprooted, washed to remove adhering soil, and observed for plant growth characteristics such as percent germination, root length, shoot length, seedling length, fresh weight, dry weight, vigor indices 1 and 2, and mortality.

Field Evaluation

Field performance of five *Pseudomonas fluorescens* isolates (Pf-173, Pf-193, Pf-547, Pf-551, and Pf-572) for BC and PGP activities were evaluated in French bean (var. *Pusa contendor*). Seventeen seed treatments (6 g kg^{-1} seed), each in three replications, were tested (Table 1).

Table 1 List of *Pseudomonas fluorescens* isolates and their combinations used in the evaluation of biocontrol and PGP activities in French Bean (var. *Pusa contendor*)

S. no.	Treatments[a]	Isolate/combinations of isolates
1	T-1	Pf-173
2	T-2	Pf-193
3	T-3	Pf-547
4	T-4	Pf-551
5	T-5	Pf-572
6	T-6	Pf-173 + Pf-551
7	T-7	Pf-193 + Pf-551
8	T-8	Pf-192 + Pf-572
9	T-9	Pf-547 + Pf-572
10	T-10	Pf-551 + Pf-572
11	T-11	Pf-173 + Pf-193 + 572
12	T-12	Pf-173 + 551 + Pf-572
13	T-13	Pf-193 + Pf-551 + Pf-572
14	T-14	Pf-547 + Pf-551 + Pf-572
15	T-15	Pf-173 + Pf-547 + Pf-551 + Pf-572
16	T-16	Pf-193 + Pf-547 + Pf-551 + Pf-572
17	T-17	Pf-173 + Pf-193 + Pf-547 + Pf-551 + Pf-572
18	Check-1	No treatment
19	Check-2	No treatment, soil inoculated with fungal pathogen

[a] All the treatments were used in plant growth promotion and biocontrol evaluation of the isolates. For plant growth promotion assays, only one check, Check-1, was used with uninoculated soil. In biocontrol assay, two checks, Check-1 and Check-2, were used. In Check-2, the top 1 kg soil in the pot was inoculated with 250 mg *Fusarium solani* f. sp. *pisi* inoculum

Ten seeds per line (line–line, 40 cm, and plant–plant, 20 cm) were sown and five lines per plot (2 m × 2 m) were maintained. Germination was recorded every day after the first seed germinated in any of the treatments and continued until complete germination was achieved in any of the treatments. Similarly, mortality was observed daily. After 30 days of germination plant growth parameters such as root length, shoot length, fresh weight, dry weight, number of root nodules per plant, and nodule weight (50 nodules) were recorded in ten randomly up rooted plants.

Data Analysis

Data generated were subjected to analysis of variance (ANOVA) using RBD/CRD to calculate the significance by magnitude of the F value ($p = 0.05$) using computer software.

RAPD-PCR Profile

Isolation and amplification of genomic DNA A set of 200 fP isolates was selected (on the basis of varied biochemical, BC, and PGP activities) for estimation of genetic diversity. Genomic DNA was isolated by following the miniprep method (Ausbel and others 1999). Template concentration was estimated with reference to calf thymus DNA on 0.8% agarose gel. Amplification reactions were carried out using 10 ng of template DNA in 20 µl of reaction mixture with 25 ng of primer, 200 µM of dNTP mix, 0.5× reaction buffer, 1.25 mM of $MgCl_2$, and 1.0 unit of *Taq* polymerase. Amplification reactions were carried out in a DNA engine (PTC-200, MJ Research, Waltham, MA, USA), with initial denaturation at 94°C for 3 min, 38 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, synthesis at 72°C for 2 min, and final extension at 72°C for 5 min. Amplified products were resolved on 1.0% agarose gel at 60 V for 2.5 h using a midi-gel assembly (Bio-Rad, Hercules, CA, USA). Gels were stained in ethidium bromide solution (0.5 µg ml^{-1}) and documented using a Gel-Doc system (Bio-Rad).

Estimation of genetic diversity Initially, 21 oligonucleotide primers were screened to assess genetic diversity among the fP isolates. On the basis of maximum polymorphism, only three primers (OPA-21: 5′ GTAGAC CCGT 3′, OPA-54: 5′ GTTTCGCTCC 3′, and OPA-75: 5′ GGTGGTCAAG 3′) were used to estimate the diversity. PCR products were resolved as explained above. Genetic diversity among the fPs was estimated on the basis of pairwise similarity coefficient and cluster analysis based on the presence and absence of bands. Computer software (Gel-Compar-II ver. 3.5, Applied Maths, St-Martens-Latem, Belgium) was used for the similarity matrix analysis and preparation of a dendrogram using the unweighted pair group method, arithmetic mean (UPGMA) algorithm with "Dice" coefficient of similarity.

Results

Biochemical Characterization

In all, 600 bacterial isolates were collected from different hosts (12 annual and 6 perennial) and locations (20 locations ranging from 1000 to 2100 masl) in Garhwal Himalayas (Table 2). Among these, 580 were positive for arginine hydrolysis, whereas all were negative for starch hydrolysis. A total of 523 isolates were found positive for gelatin hydrolysis and 386 isolates were positive for oxidase activity. A set of 318 isolates produced levan on sucrose-amended medium and were recorded positive for the trait. Of the 600 isolates that were subjected to the

Table 2 Description of different rhizospheres[a] sampled for the isolation and selection of fluorescent *Pseudomonas* isolates for the study

Host plant	No. of isolates	Soil conditions[b]			
		Temp[c] (°C)	pH	Water-holding capacity (%)	Organic matter (%)
Annuals					
Cabbage (*Brassica oleracea* var. *capitata*)	23	6–22	5.7–6.2	28.5–35.5	5.10–6.89
Cauliflower (*Brassica oleracea* var. *botrytis*)	04	12–25	5.4–6.2	16.5–35.5	4.62–6.89
Chili (*Capsicum anum*)	15	15–28	5.8–7.0	16.5–25.6	1.78–3.5
Pea (*Pisum sativum*)	74	6–22	5.7–6.2	28.5–35.5	2.5–6.89
Ginger (*Zingiber officinale*)	10	16–24	6.4–7.0	10.6–16.5	1.85–3.5
French bean (*Phaseolus vulgaris*)	121	8–21	5.4–6.2	16.5–35.5	4.62–6.89
Rice bean (*Vigna umbellata*)	09	16–22	5.4	20.5–28.0	1.9
Rice (*Oryza sativa*)	23	15–26	5.4–7.0	10.6–25.5	1.78–2.25
Ragi (*Eleusine coracana*)	23	18–26	5.4	20.5–28.0	1.9
Wheat (*Triticum aestivum*)	16	16–25	5.4	20.5–28.0	1.9
Mustard (*Brssica campestris*)	13	9.5	5.2	20.9	6.43
Amaranth (*Amaranthus tricolor*)	16	15–26	5.4–7.0	20.5–32.5	1.78–1.9
Perennials					
Apple (*Malus domestica*)	109	10–22	5.2	20–35.5	2.92
Almond (*Prunus amygdalus*)	29	10–22	6.5	20–35.5	2.86
Peach (*Prunus persica*)	62	10–22	6.8	20–35.5	3.09
Plum (*Prunus domestica*)	32	10–22	5.6	20–35.5	3.26
Pear (*Pyrus communis*)	21	10–22	6.2	20–35.5	2.92
Total	600				

[a] Rhizospheric soil of different annual and perennial hosts was collected from 20 different locations at 1000–2100 masl in Uttaranchal Himalayas

[b] Values give range of parameters as the isolates were obtained from varied farming situations

[c] Soil temperature was taken at a depth of 12 cm

ammonification test, 359 were positive for this activity (data not shown). By referring to Bergey's manual, a total of 336 rhizospheric isolates were confirmed as fluorescent pseudomonads and characterized into three major groups: *P. fluorescens*, *P. putida*, and *P. aeruginosa*. Of these, the group *Pseudomonas fluorescens* is known to consist of five biovars (biovar-I to -V). In our study, none of the isolates belonged to biovar-IV because that biovar is reported to produce blue fluorescence on KB medium, whereas the isolates in the present study were selected on the basis of yellow-green fluorescence only. Among 308 *P. fluorescens* isolates, 61 belonged to biovar-I, 114 to biovar-II, 59 to biovar-III, and 74 to biovar-V (Table 3). Results of the biochemical tests were the same for *P. fluorescens* (biovar-III) and *P. aeruginosa*, which were further differentiated on the basis of their growth at 41°C, as *P. fluorescens* (biovar-III) isolates were unable to grow at this temperature.

Growth at Different Temperatures

Results of the assay confirmed the isolates to be cold-tolerant as most isolates could grow at lower temperatures. Although none of the isolates could grow at any of the temperatures after 6 h of incubation, the majority could grow well after 18 h of incubation. A few isolates could grow even at 8°C after 12 h of incubation. Good growth in all the isolates was recorded after 18 h of incubation (data not shown). Table 4 gives the growth pattern of five Pf isolates at different temperatures and time intervals. These isolates were selected for assessment of their PGP and BC activities under polyhouse and field conditions. These five isolates can be treated as representatives of the repository of cold-tolerant fPs.

In Vitro Antagonism Against *Rhizoctonia solani*

Of the 336 isolates of fluorescent *Pseudomonas* spp., 74 inhibited the growth of *R. solani* on KB plates (Fig. 1a, Table 5). The zone of inhibition produced by the fP isolates ranged from 0.1 to 1.7 cm. Of 74 antagonists, 45 isolates were A grade and 29 B grade. The highest zone of inhibition (1.7 cm) was produced by the isolates Pf-551 and Pf-572. Percent inhibition efficacy of all the antagonists ranged between 24.88 and 85.24% (Table 5). The maximum

Table 3 Characteristics of the confirmed fluorescent *Pseudomonas* isolates

S. no.	Genus/biovar	No. of isolates	Antagonism		Performance of isolates in different tests					
			R. solani	*F. solani*	1	2	3	4	5	6
1	*P. aeruginosa*	20	6	4	+	−	+	+	−	+
2	*P. fluorescens*									
	Biovar-I	61	17	21	+	−	+	+	+	−
	Biovar-II	114	25	33	+	−	+	+	+	+
	Biovar-III	59	13	12	+	−	+	+	−	+
	Biovar-V	74	12	21	+	−	+	+	−	−
3	*P. putida*	8	1	0	+	−	−	+	−	−
Total		336	74	91						

1 arginine dihydrolysis, *2* starch hydrolysis, *3* gelatin hydrolysis, *4* oxidase reaction, *5* levan formation, *6* denitrification; +, positive reaction; −, negative reaction

Table 4 Growth of *Pseudomonas fluorescens* isolates used in the study at different temperatures and time intervals

Temp (°C)	Time (h)																								
	Pf-173					Pf-193					Pf-547					Pf-551					Pf-572				
	6	12	18	24	36	6	12	18	24	36	6	12	18	24	36	6	12	18	24	36	6	12	18	24	36
4	−	−	+	++	++	−	−	−	+	++	−	−	−	+	++	−	−	+	++	++	−	−	+	++	++
6	−	−	+	++	++	−	−	−	+	++	−	−	−	+	++	−	−	+	++	++	−	−	+	++	++
8	−	+	+	++	++	−	−	+	+	++	−	+	+	++	++	−	+	+	++	++	−	+	+	++	++
10	−	+	+	++	++	−	+	+	+	++	−	+	+	++	++	−	+	+	++	++	−	+	+	++	++
15	−	+	+	++	++	−	+	+	++	++	−	+	+	++	++	−	+	+	++	++	−	+	+	++	++
20	−	+	+	++	++	−	+	+	++	++	−	+	+	++	++	−	+	++	++	++	−	+	+	++	++
25	−	+	+	++	++	−	+	+	++	++	−	+	++	++	++	−	+	++	++	++	−	+	++	++	++

−, no growth; +, initial growth; ++, complete growth

inhibition efficacy (85.24%) was recorded for Pf-572 followed by Pf-551 (82.24%), Pf-54 (76.14%), and Pf-567 (68.86%).

Microscopic examination revealed that fP isolates inhibited the growth of fungal mycelium either by stunting the growth (static action) or through lysis of the mycelium (Fig. 1b, c). Deformities in stunted fungal mycelium were also observed (Fig. 1b). Of the 74 interactions examined, 29 were lytic and 45 were static in action (Table 5).

In Vitro Antagonism Against *Fusarium solani*

Of the 336 fPs, 91 inhibited *F. solani* growth on PDA plates (Fig. 2a, Table 5). The zone of inhibition ranged from 0.1 to 1.5 cm. Among 91 antagonistic fPs, 42 were grade A and 49 grade B. The maximum zone of inhibition (1.5 cm) was that of Pf-551 and Pf-572. Percent inhibition efficacy of all the antagonists ranged from 32.00 to 75.50% (Table 5). The highest inhibition efficacy (75.50%) was that of Pf-551 followed by that of Pf-567 (73.30%) and Pf-572 (72.22%). Microscopic examination revealed the lytic and static modes of inhibition of fP isolates (Fig. 2b, c). Deformities in fungal mycelium were again observed (Fig. 2c). Of the 91 interactions examined, 24 were lytic and 67 were static in action (Table 5).

Evaluation for HCN Production

Of the 336 fluorescent pseudomonads, 33 were positive for HCN production (Table 5). On the basis of color produced, fP isolates were placed into three categories: + (orange), ++ (brick red), +++ (brown). With increasing incubation period, the color of the filter paper tended to be darker (toward the next category), indicating an increase in HCN production with an increase in incubation period (5 and 7 days). Only Pf-23, Pf-63, Pf-81, Pf-362, and Pf-504 did not show any increase in HCN production with prolonged incubation.

Evaluation for Siderophore Production

Of the 336 fPs, 80 isolates were positive for siderophore production (Table 5). A clear orange halo was observed around bacterial growth on CAS medium. On the basis of

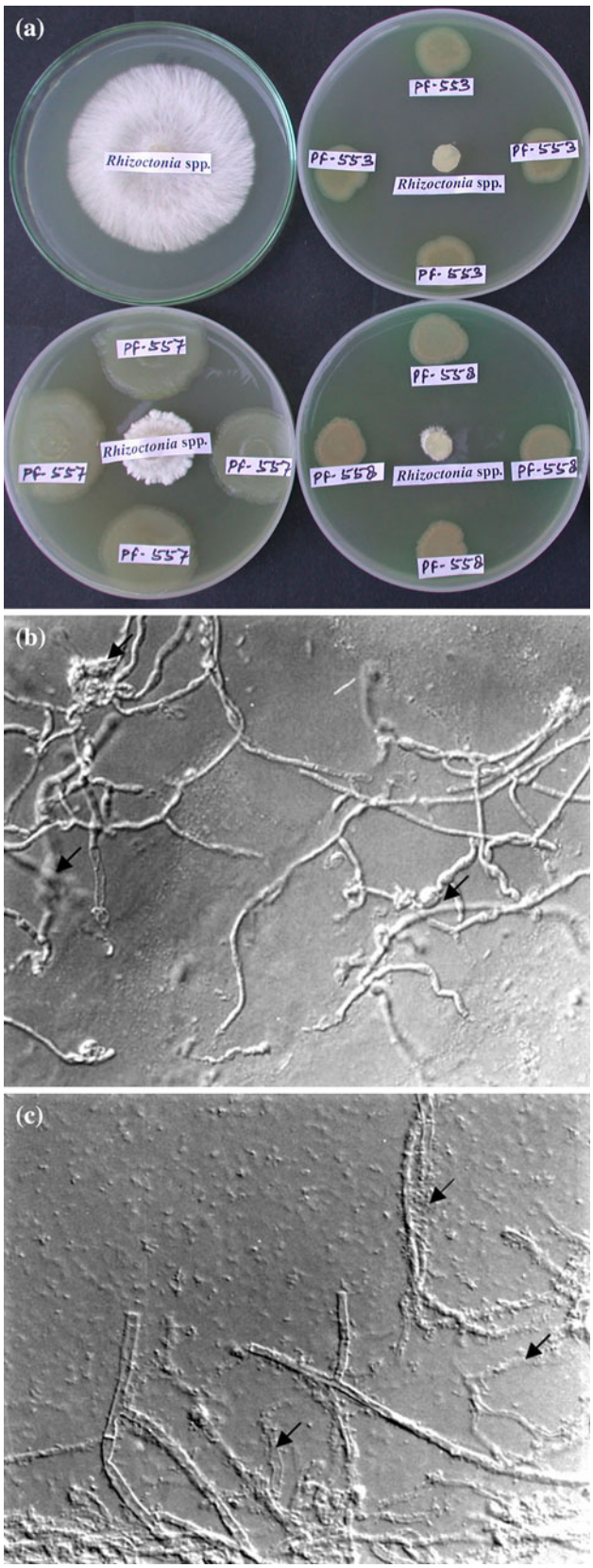

Fig. 1 **a** In vitro antagonism of fluorescent *Pseudomonas* isolates ▶ against *Rhizoctonia solani.* **b** Deformed mycelium (arrows) of *R. solani* after interaction with Pf-164. **c** Lysis (*arrows*) of mycelium of *R. solani* after interaction with Pf-551

siderophore production efficacy, all the isolates were placed into three categories: + (120–149%), ++ (150–199%), and +++ (>200%). The siderophore production efficacy of fP isolates ranged from 120 to 260%. The maximum efficacy (260%) was recorded for Pf-567 and Pf-572 followed by Pf-84 (250%) and Pf-551 (240%). Prolonged incubation resulted in more siderophore production.

Evaluation for Phosphate Solubilization

Of the 336 fPs, 49 isolates solubilized the inorganic phosphorus source to develop a zone of solubilization around the bacterial colonies (Table 5). On the basis of solubilization efficacy, fPs were placed into three categories: + (120–149%), ++ (150–199%), and +++ (>200%). The solubilization efficacy of fPs ranged from 120 to 240%. Maximum efficacy (240%) was recorded for Pf-572, followed by Pf-567 (210%) and Pf-551 (200%). Increased phosphate solubilization was observed with prolonged incubation.

Evaluation for BC and PGP Activities

Screen house assay All five Pfs showed good PGP and BC activities under controlled conditions (Tables 6, 7, respectively) as significant enhancement in different plant growth parameters was recorded. Germinated French bean seedlings exhibited enhanced root and shoot lengths in all the treatments, but the combination of five Pfs exhibited maximum planting value parameters (Table 6). Individually, isolate Pf-551 showed consistent superiority for most planting value parameters in the two experiments. Atypical symptoms (root rot, color rot, stem rot, and aerial blight) of *R. solani* infection can be seen clearly (Fig. 3e–h). A good reduction in disease development was recorded in different treatments, of which a combination of all five test isolates was found to be best. Root rot was observed to be most severe when pathogenic inoculum was mixed with pot soil. Treatments could show significant reductions (80–100%) with respect to percent mortality. The combination of all five test isolates showed 0% mortality, that is, 100% suppression of the infection.

Field evaluation Most of the treatments showed good BC and PGP activities (Table 8). Except Pf-193 (individually), all the treatments were significant for plant growth promotion. Only root rot and collar rot were observed in

Table 5 In vitro evaluation of fluorescent *Pseudomonas* isolates for biocontrol and plant growth-promoting activities

S. no.	Activity	No. of isolates[a]	% Efficacy[b]
1	In vitro biocontrol		
	a. Antagonism[c]		
	R. solani	74 (0.4–1.7)	24.88–85.24
	F. solani	91 (0.4–1.5)	32.00–75.50
	b. Mode of inhibition[d]		
	Lytic		
	R. solani	29	28.45–85.24
	F. solani	24	43.30–75.50
	Static		
	R. solani	45	24.88–76.14
	F. solani	67	32.00–66.00
2	HCN production	33	–
3	P solubilization	49	120–240
4	Siderophore production	80	120–260

–, not calculated being color-based evaluation

[a] Number of isolates showing antagonism. Figures in parentheses are range of zone of inhibition produced by the fPs against pathogens. Zone of inhibition was calculated as radius of fungal growth in control plate minus radius of fungal growth in the interaction plate after 5 days of incubation

[b] Average percent efficacy shown by the fP isolates for different traits was calculated as follows:

Antagonistic efficiency = $[(A - B)/A] \times 100$, where A is diameter of fungal growth in control plate and B is diameter of fungal growth up to zone of inhibition in the test plate

P solubilization efficacy = (diameter of zone of solubilization/diameter of bacterial growth) × 100

Siderophore producing efficacy = (diameter of zone of solubilization/diameter of bacterial growth) × 100

[c] Evaluated on King's medium B by dual-culture method

[d] Confirmed by microscopic examination and subculturing of affected mycelium

the field trial. Different treatments, except Pf-193 (individually), showed significant reduction in disease development. The combination of the five isolates proved to be best, with maximum disease reduction (93.33%) and enhancement of various plant growth parameters. Among individual isolates, Pf-551 had the best performance for most of the parameters assessed.

Estimation of Genetic Diversity

Of the 21 decamer primers screened for polymorphism, only three (OPA 21, OPA 54, and OPA 75) gave good amplification and polymorphism among the isolates. The three primers were used to evaluate a set of 200 fPs (selected on the basis of their positive reaction in different assays). Of these, 189, 180, and 192 isolates could be amplified with primers OPA 21, OPA 54, and OPA 75,

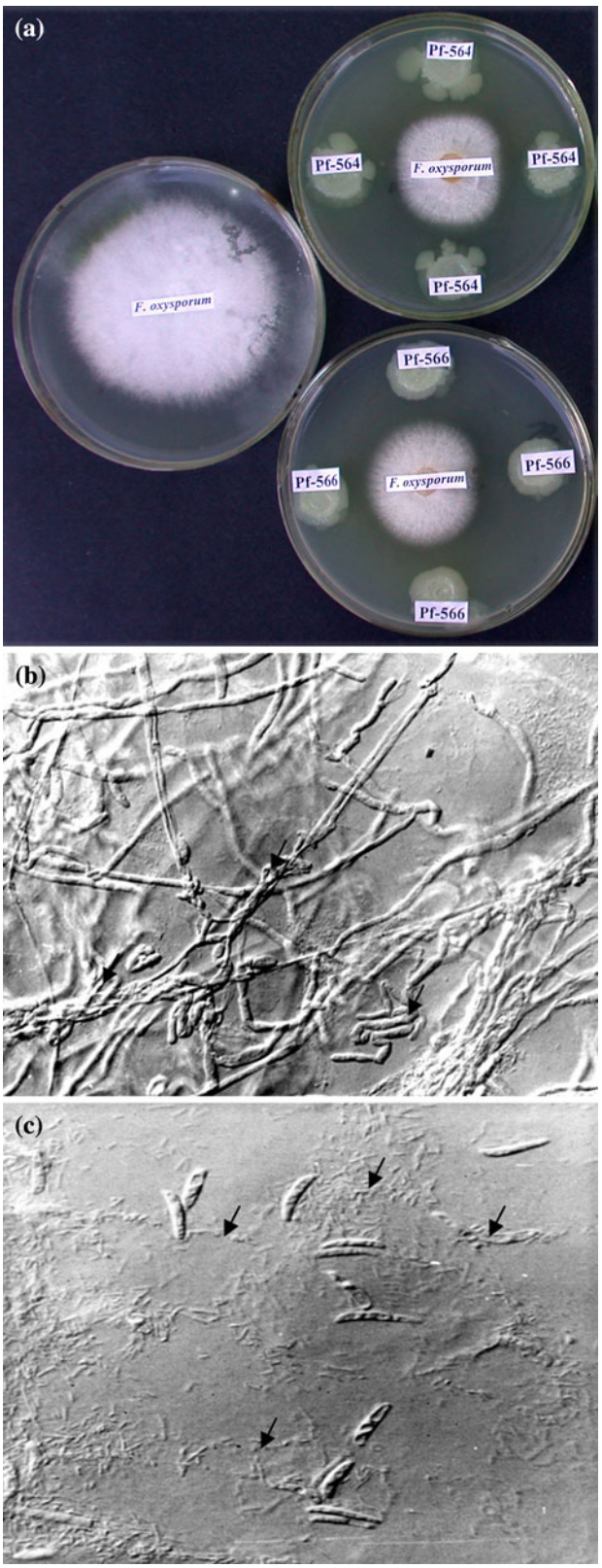

Fig. 2 **a** In vitro antagonism of fluorescent *Pseudomonas* isolates against *Fusarium solani*. **b** Deformed mycelium (*arrows*) of *F. solani* upon interaction with Pf-547. **c** Lysis (*arrows*) of mycelium of *F. solani* upon interaction with Pf-551

Table 6 Effect of seed treatment with *Pseudomonas fluorescens* isolates and their combinations on planting value parameters in French Bean (var. *Pusa contendor*) under polyhouse conditions

Treatments	Germination (%)	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Fresh wt (g)	Dry wt (g)	Nodules/ plant	Nodule wt (g)	Vigor index-1	Vigor index-2	Mortality (%)
T-1	93.33	22.51	25.35	47.86	6.80	1.16	121.00	0.10	4473.23	107.93	6.67
T-2	93.33	21.46	26.14	47.60	6.02	1.05	141.67	0.11	4450.33	98.33	10.00
T-3	100.00	22.81	25.46	48.28	5.94	1.39	175.33	0.14	4827.67	139.33	3.33
T-4	96.67	23.48	28.25	51.73	6.50	1.44	158.33	0.13	4997.93	138.93	3.33
T-5	100.00	22.22	26.20	48.42	6.52	1.41	184.33	0.15	4842.33	140.67	0.00
T-6	93.33	24.74	28.44	53.17	6.22	1.45	174.00	0.14	4964.73	135.83	6.67
T-7	100.00	24.64	29.32	53.96	6.25	1.36	155.33	0.12	5396.00	135.67	3.33
T-8	100.00	24.10	25.62	49.73	6.64	1.52	173.00	0.14	4972.67	151.67	3.33
T-9	100.00	22.20	29.69	51.89	6.68	1.34	108.67	0.09	5189.33	134.33	3.33
T-10	100.00	25.58	31.69	57.26	6.16	1.41	139.33	0.11	5726.33	141.33	0.00
T-11	96.67	25.11	27.36	52.47	6.40	1.34	121.00	0.10	5069.23	129.20	3.33
T-12	100.00	24.07	30.16	54.23	7.08	1.47	160.67	0.13	5423.33	147.33	0.00
T-13	100.00	26.25	31.52	57.78	6.96	1.53	227.00	0.18	5777.67	152.67	0.00
T-14	96.67	24.82	31.56	56.38	7.07	1.60	219.00	0.18	5463.50	154.87	10.00
T-15	100.00	27.39	32.37	59.76	7.21	1.51	202.33	0.16	5975.67	151.00	0.00
T-16	100.00	27.66	29.47	57.14	7.07	1.66	236.67	0.19	5713.67	166.00	0.00
T-17	100.00	29.26	34.00	63.26	7.40	1.76	256.00	0.20	6326.00	176.00	0.00
Check	76.67	15.88	23.55	39.43	5.05	0.83	75.33	0.08	3410.63	71.97	36.67
cd ($p = 0.05\%$)	5.96	2.10	2.44	3.46	0.31	0.141	81.16	0.06	496.21	16.07	8.73

All figures are average of three replications, each consisting of five seeds

Check = seeds receiving no treatment

cd critical difference

respectively. The number of amplified fragments ranged from 1 to 15 in the size range of 100–4000 bp. The primer OPA 75 yielded the maximum number of amplified fragments (887), with an average of 4.62.

A set of 59 representative isolates, further selected on the basis of their unique banding pattern, was again amplified with the three primers yielding a total of 316 polymorphic bands out of 840 scorable bands. The polymorphic bands detected individually with primers OPA 21, OPA 54, and OPA 75 were 73, 106, and 137, respectively (Table 9). Cluster analysis of 59 selected fPs revealed a very low level (18.6%) of similarity among the isolates and constituted two major groups (Fig. 4). Group 1 had only three isolates and the remaining 56 isolates were clustered in Group 2, which was further subdivided into nine clusters. The similarity level within and among the clusters ranged from 28.91 to 61.12% and from 40.00 to 18.60%, respectively (Fig. 4).

Discussion

On the basis of the results of biochemical tests, 336 isolates of a total of 600 were confirmed as fluorescent *Pseudomonas* and categorized into three major groups: *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Pseudomonas putida*. Among the different species and biovars, *P. fluorescens* biovar-I (bv. I) possessed the maximum number of potential candidates for BC activity against *R. solani* (27.86%) and *F. solani* (34.43%). Similarly, Nielsen and others (1998) reported a collection of 47 isolates representing all the biovars of *Pseudomonas fluorescens* from the rhizosphere of field-grown sugar beet plants. They reported the production of the lipopeptide antibiotic viscosinamide by *P. fluorescens* bv. I, whereas the antibiotic 2, 4-diacetylphloroglucinol was observed only in *P. fluorescens* bv. II/IV. Both the antibiotics were inhibitory to *Pythium ultimum* and *Rhizoctonia solani*.

In the present study a good number of antagonistic *Pseudomonas* isolates were identified on the basis of *in vitro* antagonism against both fungal pathogens. Inhibition of *R. solani* and *F. solani* by *Pseudomonas* isolates on media plates could be the result of competition for nutrients and possibly production of an antifungal metabolite (AFM) (Upadhyay and Rai 1987). Lytic or static action of different fP isolates against the two phytopathogens might be due to the type, amount, and mode of action of AFM(s) produced

Table 7 Effect of seed treatment with *Pseudomonas fluorescens* isolates and their combinations on planting value parameters and suppression of root rot disease in French Bean (var. *Pusa contendor*) grown in field soil infested with *Rhizoctonia solani* under polyhouse conditions

Treatments	Germination (%)	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Fresh wt (g)	Dry wt (g)	Nodules/ plant	Nodule wt (g)	Vigor index-1	Vigor index-2	Mortality (%)
T-1	83.33	21.07	25.97	47.04	6.23	1.21	90.33	0.08	3920.07	100.93	16.67
T-2	80.00	20.81	26.67	47.48	6.11	1.09	70.67	0.06	3798.40	87.33	20.00
T-3	90.00	21.23	27.06	48.29	6.02	1.31	86.33	0.08	4335.50	117.93	10.00
T-4	96.67	20.95	28.85	49.79	6.41	1.26	91.67	0.08	4814.97	122.50	3.33
T-5	93.33	21.09	26.87	47.96	6.61	1.27	99.67	0.09	4487.07	118.52	6.67
T-6	96.67	22.16	28.04	50.20	5.99	1.21	106.67	0.10	4855.07	117.13	3.33
T-7	93.33	21.61	28.53	50.14	5.93	1.27	125.00	0.11	4668.67	118.93	6.67
T-8	96.67	21.22	26.18	47.40	6.55	1.39	108.33	0.10	4609.67	134.60	3.33
T-9	93.33	21.96	28.91	50.87	6.71	1.28	136.00	0.12	5087.00	128.00	6.67
T-10	100.00	19.17	31.03	50.20	5.67	1.14	141.33	0.13	5020.00	114.00	0.00
T-11	100.00	21.79	27.49	49.28	6.33	1.27	132.00	0.12	4928.00	127.33	0.00
T-12	96.67	21.98	30.39	52.37	7.02	1.36	148.33	0.13	5052.50	131.94	3.33
T-13	96.67	20.72	31.86	52.59	6.85	1.39	136.67	0.12	5083.47	134.73	3.33
T-14	100.00	20.37	32.01	52.38	6.95	1.38	149.67	0.13	5238.00	138.17	0.00
T-15	100.00	20.00	33.00	53.00	6.93	1.38	140.00	0.13	5300.00	138.00	0.00
T-16	100.00	22.61	29.94	52.55	5.92	1.50	130.67	0.12	5255.33	149.83	0.00
T-17	100.00	23.65	34.37	58.02	7.26	1.57	166.33	0.15	5802.33	156.67	0.00
Check-1	53.33	11.81	19.02	30.83	3.31	0.50	42.33	0.04	1970.60	29.73	46.67
Check-2	73.33	17.33	23.45	40.78	4.86	0.71	75.33	0.07	2986.33	52.20	26.67
cd ($p = 0.05\%$)	10.50	3.48	3.96	5.79	1.21	0.22	59.04	0.05	752.05	25.39	10.40

All figures are average of three replications, each consisting of five seeds. Evaluation was done by pot test

Check-1 = untreated seeds sown in soil inoculated with the inoculum of *R. solani* at 250 mg per pot in top 1 kg soil in a 6-in.-diameter plastic pot; Check-2 = untreated seeds sown in uninoculated soil

cd critical difference

by different isolates. Several workers have reported the production of AFMs by fluorescent pseudomonads as the key factor for the inhibition of fungal pathogens. Different AFMs such as phenazine-1-carboxilic acid, pyoluteorin, 2,4-diacetylphloroglucinol, pyrrolnitrin, hydrogen cyanide, and siderophores are known to inhibit fungal pathogens (Weller 1988; Bagnasco and others 1998). In a separate study, some of the selected isolates (Pf-103, Pf-173, and Pf-193) from the same repository exhibited up to 100% inhibition of the two fungal pathogens in PDA broth, which also confirms the secretion of AFMs by fPs (Negi and others 2005).

Some of the antagonistic fPs exhibited direct inhibition of fungal growth as attachment and penetration of bacterial cells to the fungal hyphae were observed microscopically. Whereas in other cases, no bacterial cell was found to be attached to the deformed or degraded (lysed) mycelium, which suggested a direct role of secondary metabolites in inhibiting fungal growth in such cases. Lee and Kobyashi (1989) reported lysis in *R. solani*, which might be due to antifungal metabolites secreted by the antagonist strain of *Pseudomonas* (*Burkholderia*) *cepecia*. On the other hand, Chung and others (1998) and Amer and others (1997) reported mycelial deformity and lysis in *R. solani* and *F. oxysporum* due to attachment and penetration of bacterial cells into fungal hyphae.

A good range (qualitatively) of HCN production by the isolates was observed in this study. Together with other secondary metabolites, HCN production may increase the biocontrol ability of fPs. Bagnasco and others (1998) also reported the inhibition of *Pythium ultimum* and *R. solani* by *P. fluorescens* strains UP61, UP143, and UP148 with the ability to produce HCN and siderophores. Isolates in the present study showed good efficacy (120–260%) for siderophore production. Siderophore-producing fPs were found more promising in their BC activity than non-siderophore-producing strains. Seong and Shin (1996) reported that *Pseudomonas fluorescens* ps88 showed siderophore-mediated inhibition of *Pythium ultimum*, *Pyricularia oryzae*, *R. solani*, and *Xanthomonas oryzae*.

Fig. 3 Evaluation of *Pseudomonas fluorescens* isolates for biocontrol ability in French bean. **a** Inoculated check. **b** Uninoculated check. **c**, **d** Performance of different treatments against *R. solani* infection (arrows indicate check plants). **e**–**h** Different symptoms of *R. solani* infection (stem-rot in **e**, **f**, **g**; leaf blight in **e** and **f**; damping off due to root rot in **f** and **h**) in French bean (note *arrows*). Symptoms of leaf blight are also clear in **a**, **b**, **c**, and **d** set (note yellowing of leaves)

The ability of fPs to solubilize phosphate in the present study was good (120–240%). This ability of the isolates could improve phosphate uptake by the host plant. Several reports in the literature suggest using P-solubilizing PGPR for improvement of phosphate uptake by the host plant, ultimately to improve the plant health (Lifshitz and others 1987; Rodriguez and Fraga 1999). Jeon and others (2003) reported enhanced plant growth using three strains of *Pseudomonas fluorescens* (MC07, B16, and M45) of which B16 and M45 were able to produce 502.4 and 206.1 mg l^{-1} of soluble phosphate from $Ca_3(PO_4)_2$ and hydroxyapatite, respectively.

The five *Pseudomonas fluorescens* isolates (Pf-173, Pf-193, Pf-547, Pf-551, and Pf-572) used in the present study were selected on the basis of their better performance in vitro with respect to BC and PGP activities. The selected Pfs and their combinations were effective in increasing most of the planting value parameters in French bean under polyhouse and field conditions. In addition, Pf treatments yielded reduced pathogenic activity of *R. solani* in the pathogen-infested soil under polyhouse conditions and effectively reduced root rot disease by 26.67–100%. Under field conditions, infection was reduced by 28.88–73.32% in comparison to controls. Results of field trials demonstrated a sound ability of the isolates for BC and PGP activities. Plants raised from treated seeds exhibited higher planting value parameters, and percent mortality in treated plants was significantly lower than controls.

Suppression of root-rot disease clearly indicated the ability of Pfs and their combinations to antagonize the target pathogens (*R. solani*) and led to subsequent reductions in disease development. Secretion of various secondary metabolites by *Pseudomonas* spp., including production of antifungal metabolites, chitinolytic enzymes, HCN, and siderophore, has been well studied and have been reported inhibitory against different phytopathogens, including soil-borne fungal pathogens (Bakker and Schippers 1987; Weller 1988; Bagnasco and others 1998).

Enhanced seedling length and weight recorded in this study were the result of the growth-promoting abilities of *Pseudomonas* isolates. The growth-promoting ability of pseudomonads is a result of good root colonization and

Table 8 Effect of seed treatment with *Pseudomonas fluorescens* isolates on planting value parameters of French Bean (var. *Pusa contendor*) grown under field conditions

Treatments	Germination (%)	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Fresh wt (g)	Dry wt (g)	Nodules/ plant	Nodule wt (g)	Vigor index-1	Vigor index-2	Mortality (%)
T-1	81.11	8.41	19.68	28.09	6.20	1.59	85.67	0.10	2278.38	128.96	15.56
T-2	87.22	8.24	20.02	28.26	5.87	1.46	65.33	0.08	2464.84	127.34	17.78
T-3	85.00	8.78	20.57	29.35	6.77	1.61	96.00	0.12	2494.75	136.85	14.45
T-4	92.78	9.28	21.21	30.49	8.11	1.75	102.00	0.12	2828.86	162.37	11.11
T-5	89.45	8.69	20.12	28.81	7.88	1.58	120.67	0.14	2577.05	141.33	11.11
T-6	88.89	9.89	20.26	30.15	8.84	2.29	106.67	0.13	2680.03	203.56	9.45
T-7	85.56	11.81	22.13	33.94	8.47	2.27	42.33	0.05	2903.91	194.22	11.67
T-8	88.89	12.17	23.86	36.03	9.25	2.28	136.67	0.16	3202.71	202.67	12.22
T-9	89.45	12.67	23.40	36.06	8.99	2.24	124.00	0.15	3225.57	213.79	10.00
T-10	88.89	13.15	22.07	35.23	8.26	2.05	139.33	0.17	3131.59	182.22	11.67
T-11	87.78	10.35	20.71	31.07	7.92	1.98	143.33	0.17	2727.32	173.80	11.11
T-12	80.00	12.20	21.51	33.71	7.81	2.01	128.00	0.15	2696.80	160.80	10.56
T-13	87.23	13.27	24.60	37.87	9.47	2.53	134.00	0.16	3303.40	220.69	12.78
T-14	85.34	12.77	22.63	35.41	8.11	2.12	140.00	0.17	3021.89	180.92	10.00
T-15	88.89	12.64	23.46	36.09	9.33	2.11	130.67	0.16	3208.04	187.56	12.23
T-16	87.23	11.41	21.56	32.97	9.04	2.18	114.00	0.14	2875.97	190.16	7.22
T-17	93.89	13.45	25.27	38.73	9.73	2.39	166.33	0.20	3636.36	210.31	6.67
Check	73.89	6.99	14.76	21.75	4.72	0.96	30.00	0.04	1607.11	70.93	25.00
cd ($p = 0.05$ %)	11.95	1.54	2.26	3.03	1.36	0.52	45.31	0.05	517.57	17.09	8.89

All figures are average of three replications. Planting value was evaluated in plots (2 m × 2 m). Seeds were treated with talc formulation of Pfs

Check = seeds receiving no treatment

cd critical difference

expected production of growth hormones like auxins, gibberellins, and cytokinins, which have been shown to be produced by different fluorescent *Pseudomonas* strains (Digat and others 1984; Young and others 1991; Glick 1995). Plant vigor is an indicator of the ability of the crop/host plant to withstand adverse conditions. In different experiments, plant vigor was effectively increased with different treatments. Under field conditions, plant vigor was significantly increased over controls by different treatments (except Pf-193) in the present study.

A combination of the five test Pfs was better than individual isolates with respect to BC and PGP activities. A consortium of different strains has been shown to give enhanced performance in previous studies also (Pierson and Weller 1994; Duffy and Weller 1995). Greater diversity of introduced bacterial inoculants results in a diverse but potentially more stable rhizosphere community to colonize the root system. Furthermore, diverse populations of PGPRs increase the spectrum of action because different strains possess different modes of action (Guetsky and others 2001; De Boer and others 2003). Application of a mixture of PGPR strains would more closely mimic the natural situation and might broaden the spectrum of action to enhance the efficacy and reliability of control (Duffy and Weller 1995). It is thus reasonable to suggest that manipulation of the composition of microorganisms in the rhizosphere with sustainable effects could be best accomplished by introducing mixtures of compatible microorganisms. Such effective combinations should reduce the use of hazardous chemicals and their adverse effects on ecosystems.

In the present study, the fluorescent *Pseudomonas* population collected from the Garhwal hills of Uttarakhand Himalayas was first characterized for some specific biochemical tests and biocontrol efficacy against two major fungal pathogens. Results of the characterization gave baseline information on the amount of diversity that exists among fluorescent pseudomonads. The set of three primers revealed groupings of isolates with respect to host of origin and habitat. Except for a few cases, the set of three primers largely grouped the isolates on the basis of their host/rhizosphere. Most of the isolates, which were collected from the rhizospheres of apple, almond, pear, and plum (for example, Pf-225, Pf-255, Pf-256, Pf-267, and 290), were grouped in subcluster I of cluster VIII. Isolates Pf-03, Pf-04, Pf-05, Pf-07, Pf-11, Pf-86, and Pf-490, which originated from the rhizosphere of annual plants (pea, cabbage, and wheat), were grouped in cluster IV (Fig. 4). Clusters III and V also possessed isolates from different annual

Table 9 RAPD-PCR amplification of 59 selected fluorescent *Pseudomonas* isolates with three oligodecamer primers

Isolate	Amplification with primer			Total no. of bands[a]	Total no. of polymorphic bands with primer			Total no. of polymorphic bands[b]	% Polymorphism[c]
	OPA-21	OPA-54	OPA-75		OPA-21	OPA-54	OPA-75		
Pf-3	2	3	6	11	0	0	1	1	9.09
Pf-4	5	4	6	15	0	0	2	2	13.33
Pf-5	4	3	6	13	0	1	1	2	15.38
Pf-7	5	2	5	12	1	1	3	5	41.67
Pf-11	5	3	6	14	0	0	1	1	7.14
Pf-14	2	5	5	12	0	1	1	2	16.67
Pf-17	4	4	6	14	2	3	2	7	50.00
Pf-68	4	3	10	17	2	2	5	9	52.94
Pf-72	2	3	3	8	2	2	0	4	50.00
Pf-84	3	3	4	10	2	2	1	5	50.00
Pf-86	5	6	6	17	1	2	3	6	35.29
Pf-95	3	4	2	19	2	2	0	4	44.44
Pf-103	3	5	3	11	0	2	3	5	45.45
Pf-125	4	3	6	13	1	1	3	5	38.46
Pf-136	3	5	3	11	0	2	1	3	27.27
Pf-149	5	4	5	14	3	3	3	9	64.28
Pf-164	5	5	4	14	3	3	2	7	50.00
Pf-173	6	3	4	13	3	0	1	4	30.77
Pf-191	8	4	5	17	3	0	1	4	23.53
Pf-195	6	6	4	16	2	3	3	8	50.00
Pf-225	7	7	5	19	3	4	2	9	47.37
Pf-236	7	6	4	17	2	5	2	9	52.94
Pf-255	4	8	6	18	2	4	3	9	50.00
Pf-256	6	6	4	16	1	2	2	5	31.25
Pf-267	7	10	10	27	4	5	5	14	51.85
Pf-290	6	7	7	20	4	3	3	10	50.00
Pf-295	4	7	6	17	0	5	3	8	47.06
Pf-364	6	7	5	18	2	2	3	7	38.89
Pf-379	5	6	9	20	1	4	4	9	45.00
Pf-396	5	4	7	16	1	1	4	6	37.50
Pf-401	2	3	5	10	0	1	3	4	40.00
Pf-410	3	6	10	19	0	2	4	6	31.58
Pf-416	5	2	5	12	2	1	3	6	50.00
Pf-428	4	6	6	16	1	0	2	3	18.75
Pf-490	4	2	7	13	3	1	3	7	53.85
Pf-503	2	5	5	12	0	1	1	2	16.67
Pf-542	3	2	3	8	2	0	0	2	25.00
Pf-547	3	5	7	15	1	1	2	4	26.67
Pf-548	3	5	9	17	1	2	4	7	41.18
Pf-549	3	4	9	16	0	2	7	9	56.25
Pf-551	3	2	7	12	1	0	3	4	33.33
Pf-552	4	5	7	16	1	1	3	5	31.25
Pf-557	3	1	5	9	1	0	3	4	44.44
Pf-558	2	4	6	12	0	0	3	3	25.00
Pf-563	3	5	7	15	0	3	4	7	46.67

Table 9 continued

Isolate	Amplification with primer			Total no. of bands[a]	Total no. of polymorphic bands with primer			Total no. of polymorphic bands[b]	% Polymorphism[c]
	OPA-21	OPA-54	OPA-75		OPA-21	OPA-54	OPA-75		
Pf-566	2	2	6	10	0	0	3	3	30.00
Pf-567	3	5	2	10	0	2	2	4	40.00
Pf-568	3	4	5	12	0	0	2	2	16.67
Pf-570	2	6	2	10	2	3	2	7	70.00
Pf-572	5	8	7	20	1	3	1	5	25.00
Pf-574	2	2	6	10	0	2	2	4	40.00
Pf-584	7	4	4	15	1	2	2	5	33.33
Pf-585	5	7	6	18	2	2	3	7	38.89
Pf-588	2	8	3	13	0	4	2	6	46.15
Pf-589	2	7	5	14	1	3	1	5	35.71
Pf-590	6	5	4	15	1	2	0	3	20.00
Pf-592	4	3	4	11	2	1	1	4	36.36
Pf-594	4	2	3	9	1	0	0	1	11.11
Pf-596	4	5	3	12	2	2	3	7	58.33
Total	239	271	320	840	73	106	137	315	

[a] Cumulative bands produced by the three primers

[b] Cumulative polymorphic bands produced by the three primers

[c] % Polymorphism = (total no. of bands/total no. of polymorphic bands) × 100

hosts. Similarly, subcluster II of cluster VIII of group 2 and group 1 possessed isolates from similar farming situations, that is, C-block and Chunnipani, respectively. Cluster IV and subcluster II of cluster VIII possessed isolates from close or similar farming situations, suggesting habitat-specific grouping (Fig. 4). A low level of similarity revealed with three primers could be because of the diverse origin of the isolates. Furthermore, the isolates that were subjected to diversity assessment were the set of selected isolates with different traits. A higher diversity level among the pseudomonads was also reported by Raaijmakers and Weller (2001) and Ramesh Kumar and others (2002). Our study also strengthens the use of RAPD-PCR as an effective and rapid technique for estimating diversity among the PGPRs. The set of the different random primers can also increase the specificity of the groups. Mavrodi and others (2001) estimated genetic diversity among 123 *P. fluorescens* using RAPD-PCR, BOX-PCR, and correlated identification of 2, 4-DAPG-producing strains on the basis of the *phlD* gene by RFLP analysis. Similarly, McSpadden Gardener and others (2000) developed a rapid and reliable PCR-based assay for rapid characterization of the 2,4-DAPG-producing *Pseudomonas* population based on the amplification of the *phlD* gene sequence.

Our work is underway to identify/develop ideal strain(s) or a consortium that could effectively exhibit BC and PGP activities in the major cash crops in different farming situations of the region. One of the important parts of the present study is the finding of the better field performance of the isolates. The field trial was conducted in February and the soil temperature varied from 7.5 to 11.5°C during the study. Although the temperature was quite low, different treatments showed a significant difference with respect to different planting value parameters and percent mortality due to *R. solani* infection. Similar findings were recorded in another study (Negi and others 2008) in which the same isolates with some more combinations were tested with pea under field conditions at a different farming locations. Soil temperature was recorded between 2 and 4°C during the study, but a good effect of the isolates and combinations on planting values was shown in the study. In both experiments, the isolates indicated and proved their cold-tolerant nature, which was expected as they were isolated from the same or nearby farming situations. The explanation we found for their success, even under adverse field conditions, was because they were used in the same environment from which they were isolated.

The repository thus created entails diverse fP isolates that could be exploited under field conditions periodically to select suitable candidates over time and space which would help in strengthening the plant growth-promoting and biocontrol activities and a more succinct disposition of the candidates in the ecosystem.

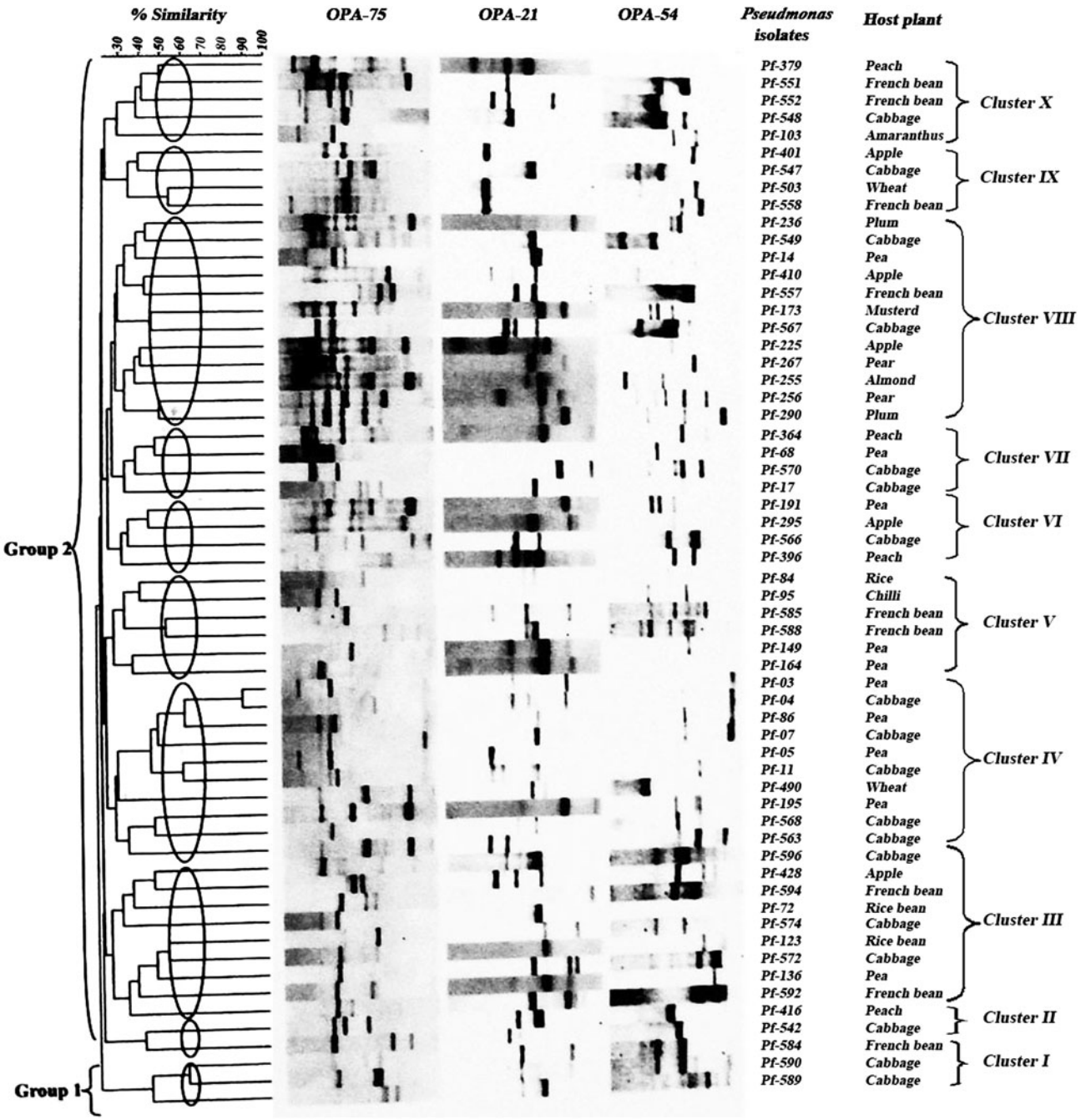

Fig. 4 Random amplified polymorphic DNA cluster analysis of fingerprint patterns generated with a set of three oligodecamer primers (OPA-21, OPA-54, and OPA75) from genomic DNA of 59 selected isolates of fluorescent *Pseudomonas* spp. Two independent amplifications were done for each isolate. The unweighted pair-group method, arithmetic mean (UPGMA) algorithm was applied to the similarity matrix generated by Dice coefficient

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