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Isolation and characterization of *Pseudomonas fluorescens* from forest soils of Uttar Karnataka with high regeneration

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Abstract
Generally, forests represent largely productive or regenerative habitats that act as carbon storage systems. In such habitats the organic matter of soil is formed as a result of decay of biomass residue along with the carbon that is deposited in the rhizosphere. Forests represent a high position of spatial diversity. Fungi, bacteria and archaea inhabit various forests. *Bacillus* and *Pseudomonas* species are well known for effectively solubilizing nutrients that limit plant growth like phosphate and potassium which ultimately improved plant growth. These bacteria colonize the plant rhizosphere and increase the plant growth by various mechanisms. The use of beneficial microorganisms as biofertilizers is increasing in agriculture and also helps to replace or reduce chemical-based fertilizers as well as pesticides usage. In the present study, *Pseudomonas fluorescens* were isolated and characterized from the soil samples of naturally regenerative ecosystems like forests in order to identify fluorescent pseudomonad isolates which are well known as plant growth promoting rhizobacteria.

Keywords: Biocontrol agents, biofertilizers, PGPR, regenerative ecosystems, Rhizodeposited carbon

1. Introduction
Elliott and Lynch (1994) [4] referred to healthy soil as a stable soil with tolerance to stress, rich in biodiversity and the maximum level of internal cycling of nutrients. Similarly, ecosystem stability is linked to biodiversity and adaptability to stress. The definition of soil resilience is tolerance to stress, buffering capacity and the ability to regenerate. Generally, forests represent largely productive or regenerative habitats that act as carbon storage systems. In such habitats the organic matter of soil is formed as a result of decay of biomass residue along with the carbon that is deposited in the rhizosphere. Forests represent a high position of spatial diversity. Fungi, bacteria and archaea inhabit various forests. Haas and Defago (2005) [6] reported that a group of typical, non-pathogenic saprophytes that colonise soil, water and plant surfaces are referred to as *Pseudomonas fluorescens*. They are rod-shaped, Gram –ve bacteria that secretes fluorescein, a soluble greenish fluorescent pigment, as its name suggests, especially when there is little iron available. Except for few strains that can use NO_3 as an electron acceptor instead of O_2, it is an obligate aerobe. Fluorescent Pseudomonas is a member of the PGPR, which is important for promoting plant development, inducing systemic resistance and biologically controlling pathogens. The ability of antibiotics and siderophores generated by bacteria to control phytopathogens could be very important.
Pseudomonas fluorescens strains are among the commercially available biocontrol organisms that are frequently employed in agriculture to reduce soil-borne plant diseases and improve plant health and productivity. According to Spaepen *et al*. (2007) [10], indole-3-acetic acid, an active plant hormone in soil is secreted by beneficial bacteria in the soil such as *Bacillus* spp. and *Pseudomonas* spp., and it has been shown to have promising characteristics improving soil quality. By supplying nutrients, soil beneficial bacteria encourage fertility in the rhizosphere, which is a significant process. According to Chaudhary *et al*. (2021) [3], *Bacillus* and *Pseudomonas* species are well known for effectively solubilizing nutrients that limit plant growth like phosphate and potassium which ultimately improved plant growth. Therefore, the present investigation involved the isolation as well as characterization of *Pseudomonas fluorescens* from forest soils of Uttar Karnataka with high regeneration.

2. Materials and Methods
2.1 Soil sampling

The soil samples from rhizosphere were collected from different forest types like evergreen, deciduous and degraded patches of natural forests of Haliyal, Dandeli, Gutti, Joida, Mirjan-Ramnagar road regions of Karnataka. Later, the top litter layer of the soil (two cm) was removed and then the soil samples were collected, packed in fresh polythene covers, labeled and then stored at 4 ºC to maintain the viability of organisms.

2.2 Isolation and purification of *Pseudomonas fluorescens*

The *Pseudomonas fluorescens* spp. were isolated from forests soils by serial dilution & plate count technique. The 0.1 ml of soil suspension from 10^{-4} dilution was spread onto King's B media plates & incubated at 28±2 ºC for 48 hours. Later, plates were observed for the fluorescence under UV illuminator. Such colonies were purified by four-way streaking. The single colonies from four-way streaked plates were inoculated to King's B agar slants & stored for further studies.

2.3 Morphological Characterization

Morphological characteristics of 25 fluorescent pseudomonad isolates were done based on cell shape, colour and Gram reaction (Graham and Parker, 1964) [5].

2.4 Biochemical Characterization

The biochemical characteristics of the fluorescent pseudomonad isolates were done according to the standard protocols.

2.4.1 Starch Hydrolysis

Overnight grown cultures in broth (10 µl) were spotted on to sterile starch agar plates & incubated at 28±2 ºC for 24 to 48 hours. Later, the plates were flooded with iodine solution and the appearance of transparent zone around the colony was considered as +ve reaction for the test.

2.4.2 Indole Production

Sulfide indole motility agar slants were inoculated with the overnight grown cultures of the fluorescent pseudomonad isolates & incubated at 28±2 ºC for 48 hours. Later, ten drops of Kovac's indole reagent were added to each tube and observed for red colour. Appearance of red colour were considered as +ve for indole test (MacWilliams, 2009) [7].

2.4.3 Catalase Test

Pure cultures of fluorescent pseudomonad isolates were taken on a clean glass & one or two drops of 30 per cent H2O2 was added. Formation of effervescence is considered as positive for catalase test.

2.4.4 Oxidase Test

A filter paper was dipped in 1 per cent Kovacs oxidase reagent & let to dry. Fresh pure cultures of fluorescent pseudomonad isolates were rubbed onto treated filter paper and checked for variations in colour. When the colour changes to dark purple within 5 to 10 seconds the test isolates were considered oxidase +ve (Cappuccino & Sherman, 1996) [2].

2.4.5 Gelatin liquefaction

The overnight pure cultures of the fluorescent pseudomonad isolates were inoculated to sterilized nutrient gelatin deep tubes and incubated at 28±2 ºC for 24 hours. Later, they were refrigerated for 30 minutes at 4 ºC. After refrigeration, the isolates that showed liquification of gelatin were considered +ve for the test and the ones that solidified gelatin were –ve for the test (Bradbury, 1970) [1].

2.4.6 Methyl Red Test

Test tubes containing autoclaved glucose phosphate broth were inoculated with the fluorescent pseudomonad cultures and incubated at 28±2 ^{0}C for 48 hours. Later, 5-6 drops of methyl red indicator was added and appearance of red colour production was considered positive. Whereas yellow colour production was considered as –ve for the test (Seeley & Vandemark, 1981) [9].

2.4.7 Voges Prausker's Test

Test tubes containing autoclaved glucose phosphate broth were inoculated with the fluorescent pseudomonad cultures and incubated at 35±2 ^{0}C for 48 hours. Later, 10 drops of Baritt's reagent was added & appearance of pink colour in the broth was considered as +ve for the test (Seeley & Vandemark, 1981) [9].

2.4.8 Citrate Utilization

Simmon's citrate agar slants were inoculated with overnight cultures & incubated at 28±2 ^{0}C for 24 hours. Colour change from green to blue were taken as +ve for the test (Seeley & Vandemark, 1981) [9].

3. Results and Discussion

3.1 Collection of soil samples from forests

The soil samples were collected from different forests types like evergreen, deciduous and degraded patches of natural forest of Uttar Karnataka in polyethylene cover. The details of the sources were recorded using the GPS systems and presented in Table 1.

3.2 Morphological and Biochemical Characterization

A total of 25 *Pseudomonas fluorescens* were isolated from different forest types like evergreen, deciduous and degraded patches of Uttar Karnataka. The bacterial colonies exhibiting fluorescence on King's B medium were isolated, sub-cultured and maintained on the nutrient agar slants for further experiments. The purified isolates thus obtained were characterized according to the specific characters described in Bergey's Manual of Systematic Bacteriology.

The 25 *Pseudomonas fluorescens* isolates were morphologically characterized based on the colony shape, cell shape, colour, elevation, surface, pigmentation and Gram reaction. For all the isolates the gram reaction was negative and the shape of the cell was rod. The shape of the colony of the isolates were found to be round, irregular, colour was found to be white, dull white, off white and yellowish green. Elevation was found to be convex, flat and raised. Surface was smooth, shiny and mucoid. Margin was regular and irregular. Pigmentation varied from bluish green, light green to yellowish green. The details of morphological studies are presented in table 2.

Later, these 25 isolates were biochemically characterized by conducting the biochemical tests. The results depicted that all 25 isolates were positive for citrate utilization test and negative for VP test. Whereas one isolate showed positive result for indole test, 13 isolates were positive for MR test, 20 isolates were positive for catalase test, 21 isolates weere

positive for oxidase test, starch hydrolysis and 5 isolates were positive for gelatin hydrolysis test (Table 3).

Our results agree with Manasa *et al.* (2017) [8] who isolated and characterized *Pseudomonas fluorescens* isolates from different rhizosphere soils of Telangana. Morphological identification of *Pseudomonas fluorescens* isolates were done by observing colony shape, size, colour, cell shape, colour and Gram reaction. The isolates were small and medium sized along with smooth and shining surface. Among fifteen isolates, six isolates were yellowish green in colour with light green pigmentation and other nine isolates were dull white in colour without any pigmentation. They were Gram -ve, small rods with no sporulation. Biochemical and physiological characterization of *Pseudomonas fluorescens* isolates showed +ve results for catalase as well as oxidase tests while they were -ve for Voges Prausker's test. In case of methyl red test MP-1, MP-2, RGP-1, RP-1, RP-4, SFP-1 and SFP-2 isolates were positive. Among fifteen isolates only twelve were positive for starch hydrolysis test, only three isolates were for gelatin liquefaction, twelve were positive for citrate utilization, only five isolates were positive for H_2S test, denitrification test.

Suman *et al.* (2015) [11] isolated, characterized native *Pseudomonas fluorescens* isolates from Rangareddy district, Telangana and studied their colony shape, colour, size, cell shape, colour, Gram reaction, pigmentation and formation of spore. The results showed that all the isolates developed were small to medium in size, smooth shining colonies, convex elevation, Gram -ve rods with-out any spore formation. The pure cultures of the isolates were observed under UV light for fluorescence and the results showed yellowish green, dark green, bluish green fluorescence and most of the isolates showed light green fluorescence. The results are in line with Wasi *et al.* (2010) [12] who isolated strain SM1 and characterized morphologically, biochemically and tentatively identified as *Pseudomonas fluorescens*.

Table 1: Details of soil samples collected from various forest types

Location	Forest type	GPS coordinates			*Pseudomonas fluorescens* isolate code
		Latitude (N)	Longitude (E)	Elevation (Above msl)	
Haliyal	Deciduous	15°18'29"	74°41'43"	483	HFPI-20, 21, 22
Haliyal	Deciduous	15°18'29"	74°41'43"	489	HFPI-23, 24, 25
Mirjan-Ramnagar road	Degraded	15°18'28"	74°41'43"	486	MFPI-15, 16, 17
Mirjan-Ramnagar road	Degraded	15°18'29"	74°41'43"	487	MFPI-18, 19
Dandeli	Deciduous	15°12'22"	74°38'40"	471	DFPI-11, 12
Dandeli	Deciduous	15°12'22"	78°38'40"	472	DFPI-13, 14
Gutti	Deciduous	15°8'57"	74°40'59"	461	GFPI-6, 7, 8
Gutti	Deciduous	15°8'58"	74°48'58"	452	GFPI-9, 10
Joida	Evergreen	15°9'54"	74°28'34"	625	JFPI-1
Joida	Evergreen	15°9'54"	74°28'34"	581	JFPI-2
Joida	Evergreen	15°9'55"	74°28'34"	572	JFPI-3
Joida	Evergreen	15°9'45"	74°28'33"	572	JFPI-4, 5

Note: GPS: Global Positioning System

Table 2: Morphological characteristics of fluorescent pseudomonad isolates

Sl. No.	Isolate code	Colony shape	Colour	Elevation	Surface	Margin	Pigmentation	Gram reaction	Shape
1	JFPI-1	Round	Dull white	Convex	Smooth, shiny	Regular	Bluish green	-	Rod
2	JFPI-2	Round	Yellowish green	Convex	Smooth, shiny	Regular	Light green	-	Rod
3	JFPI-3	Round	White	Flat	Smooth	Irregular	Bluish green	-	Rod
4	JFPI-4	Round	Yellowish green	Raised	Smooth, mucoid	Regular	light green	-	Rod
5	JFPI-5	Round	Off white	Convex	Smooth, shiny	Regular	Light green	-	Rod
6	GFPI-6	Irregular	Yellowish green	Convex	Smooth	Irregular	Yellowish green	-	Rod
7	GFPI-7	Round	Yellowish green	Convex	Smooth, shiny	Regular	Yellowish green	-	Rod
8	GFPI-8	Irregular	Yellowish green	Convex	Smooth, shiny	Regular	Yellowish green	-	Rod
9	GFPI-9	Irregular	Yellowish green	Flat	Smooth	Regular	Yellowish green	-	Rod
10	GFPI-10	Round	Yellowish green	Convex	Smooth, shiny	Irregular	Yellowish green	-	Rod
11	DFPI-11	Round	Yellowish green	Convex	Smooth, shiny	Regular	Light green	-	Rod
12	DFPI-12	Irregular	White	Convex	Smooth, shiny	Regular	Light green	-	Rod
13	DFPI-13	Round	Off white	Convex	Smooth, shiny	Regular	Light green	-	Rod
14	DFPI-14	Round	Yellowish green	Convex	Smooth, shiny	Regular	Light green	-	Rod
15	MFPI-15	Irregular	Dull white	Convex	Smooth, shiny	Irregular	yellowish green	-	Rod
16	MFPI-16	Round	Off white	Flat	Smooth	Irregular	Light green	-	Rod
17	MFPI-17	Round	White	Convex	Smooth, shiny	Regular	yellowish green	-	Rod
18	MFPI-18	Round	Dull white	Raised	Smooth, mucoid	Regular	Yellowish green	-	Rod
19	MFPI-19	Irregular	Off white	Flat	Smooth	Irregular	Bluish grren	-	Rod
20	HFPI-20	Irregular	White	Convex	Smooth, shiny	Regular	yellowish green	-	Rod
21	HFPI-21	Round	Yellowish green	Convex	Smooth	Irregular	Light green	-	Rod
22	HFPI-22	Round	Yellowish green	Convex	Smooth, Shiny	Regular	yellowish green	-	Rod
23	HFPI-23	Round	Yellowish green	Raised	Smooth, mucoid	Regular	Light green	-	Rod
24	HFPI-24	Round	White	Convex	Smooth, Shiny	Regular	Yellowish green	-	Rod
25	HFPI-25	Round	Yellowish green	Convex	Smooth, Shiny	Regular	Light green	-	Rod

Table 3: Biochemical characteristics of fluorescent pseudomonad isolates

Sl. No.	Isolate code	Indole test	MR test	VP test	Catalase test	Oxidase test	Citrate utilization	Starch hydrolysis	Gelatin hydrolysis
1	JFPI-1	-	-	-	+	+	+	+	+
2	JFPI-2	-	+	-	-	+	+	+	-
3	JFPI-3	-	+	-	+	+	+	+	-
4	JFPI-4	-	-	-	+	+	+	+	-
5	JFPI-5	-	-	-	+	+	+	+	-
6	GFPI-6	-	+	-	+	+	+	+	-
7	GFPI-7	-	+	-	+	-	+	+	-
8	GFPI-8	-	+	-	+	+	+	-	+
9	GFPI-9	-	-	-	+	+	+	+	-
10	GFPI-10	-	+	-	+	+	+	+	-
11	DFPI-11	+	+	-	+	+	+	-	-
12	DFPI-12	-	-	-	-	-	+	+	-
13	DFPI-13	-	-	-	-	+	+	+	+
14	DFPI-14	-	-	-	+	-	+	-	-
15	MFPI-15	-	-	-	+	+	+	+	-
16	MFPI-16	-	-	-	+	+	+	+	-
17	MFPI-17	-	+	-	+	+	+	+	-
18	MFPI-18	-	+	-	+	-	+	+	-
19	MFPI-19	-	+	-	+	+	+	-	+
20	HFPI-20	-	-	-	+	+	+	+	+
21	HFPI-21	-	+	-	-	+	+	+	-
22	HFPI-22	-	+	-	+	+	+	+	-
23	HFPI-23	-	-	-	+	+	+	+	-
24	HFPI-24	-	-	-	+	+	+	+	-
25	HFPI-25	-	+	-	-	+	+	+	-

4. Conclusion

These isolates should be further screened for their regenerative traits by conducting pot experiment and then by field experiments in order to know their role in regeneration of soil fertility and health. The current investigation data revealed that *Pseudomonas fluorescens* isolates JFPI-2 and GFPI-9 have the ability to improve soil health as well as plant growth which should be studied further by conducting pot and field experiments.

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