

Morphological and Physiological Diversity of Native Fluorescent Pseudomonads from Eastern India

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Authors' contributions

This work was carried out in collaboration among all authors. Author AKG conducted the research work and compiled the literature for manuscript. Authors Erayya and AS together have designed the study wrote the protocol and the first draft of the manuscript. Author NV managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

The potential negative effect of agrochemicals on the global environment and the cost associated with production lead to research for replacing the chemical fertilizers with bacterial inoculants. *Pseudomonas fluorescens* an important biocontrol agent because of its ability to induce plant growth and fungicidal/fungistatic activity. The native population of these rhizobacteria plays an important role in sustainable agriculture as they majorly dominate the rhizosphere. In the present study native, twenty-six fluorescent Pseudomonad cultures were isolated from the rhizospheric soil samples collected from the different locations of Zone 3A, Bihar and evaluated for their morphological and physiological diversity. Among the sixteen fluorescent pseudomonads tested, twelve of them produced round to oval colonies on Kings B medium. Of the sixteen isolates ascertained WHSB was produced maximum (51.33×10^{-8}) number of colonies on Kings B medium followed by MSSB (28.33×10^{-8}), BRKH (26.67×10^{-8}) and TMSB (26.00×10^{-8}). Fluorescent pseudomonad isolates varied

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slightly in their colony colour like green, dull-white, whitish-yellow and yellow on culture medium, the pH of 7.5 and glucose as a carbon source found best for the growth of fluorescent pseudomonads. The present work may use to develop effective formulations of potential fluorescent pseudomonads for management of soil-borne plant pathogens.

Keywords: Diversity; fluorescent pseudomonads; morphology; native population.

1. INTRODUCTION

The irrational use of chemical fertilizers and pesticides cause incredible harm to the environment. These chemicals are hazardous as well as persistently accumulate in nature. But, the imminent challenge of feeding the growing population is forcing as to follow intensive agricultural practices which aim to reduce pesticide load without being compromised for productivity. An answer to this problem is replacing chemicals with biological approaches, which are considered more environment-friendly in the long term. Biological control of plant pathogens by antagonistic microorganisms is a potential non-chemical means [1] and is known to be a cheaper, effective and eco-friendly method for the management of crop diseases [2].

The market share of microbial biopesticides is very poor and is currently occupying about 4% of the total pesticide market share in India. For increasing the use of biocontrol agents by farmers, the bottlenecks should be identified and immediate attention may be given to overcome them [3]. Till now 452 biocontrol agents are used in the production of over 2000 commercial formulations worldwide. It includes 149 microorganisms, 89 natural products, 140 insects and 74 semiochemicals [4].

Biological control agents are being supplied in India by private companies (80), ICAR Institutes (8), SAU's (10) and Central Integrated Pest Management Centres (30) [5]. At present, a total of 14 bacteria and 12 fungi have been registered with the EPA for the control of plant diseases. Most of these are sold commercially as one or more products. The technology of commercialization is still in its initial phase. 65% of the EPA registered organisms have been registered within the past 10 years while the remaining 36% registered over the past five years [6].

The genus *Pseudomonads* encompasses arguably the most diverse and ecologically significant group of bacteria on the planet and found in large numbers in the natural environment. This universal distribution suggests

a remarkable degree of physiological and genetic adaptability of *Pseudomonads* to different crops [7]. *Pseudomonad* species have been widely studied as biological control agents (BCAs) and it is alternative to the application of chemical fungicides [8].

Mechanism of biological control of plant pathogens by fluorescent *Pseudomonads* generally involves competition for nutrients, production of bacterial metabolites such as iron-chelating siderophores, hydrogen cyanide (HCN), antibiotics, extra-cellular lytic enzymes and induced systemic resistance [9,10]. The biocontrol mechanism to suppress fungal pathogens by *Pseudomonas* spp. normally involves the production of antibiotics and has a gene cluster that produces a suite of antibiotics, including 2,4 diacetyl phloroglucinol (DAPG), phenazine, pyrrolnitrin, pyoluteorin and biosurfactant antibiotics [11].

Fluorescent *Pseudomonads* are uniquely capable of synthesizing many of these antibiotics, not only to enhance its fitness but also helps in the maintenance of soil health and protection of crops from pathogens [12]. Fluorescent *Pseudomonads* have been successfully used for biological control of several plant pathogens [13] and biological control using PGPR strains especially from the genus *Pseudomonads* is an effective substitute for chemical pesticides to suppress plant diseases [14]. A prerequisite for introducing biological control agents in the environment is generally that, in addition to effective disease suppression, the effects on non-target organisms should be negligible.

Thus, native bacteria present in the same ecological niche as the pathogen must be used as biological control agents [15]. Competition for nutrients and niches is a fundamental mechanism by which an effective biocontrol agent can protect plants from phytopathogens (Singh, 2013). In the present study keeping in view the importance of fluorescent *Pseudomonad* as a successful biocontrol agent, they have been isolated from the rhizosphere of different crops from different locations of Zone

3A (South alluvial plain zone) and Kishanganj district of Bihar and evaluated for their morphological and physiological diversity.

2. MATERIALS AND METHODS

Soil samples were collected from rhizospheric soils of different crops at different locations of zone 3A and Kishanganj district, Bihar, India. Standard soil sampling protocol was followed while collecting the soil samples. The fresh samples were used for the isolation of fluorescent Pseudomonads on King's-B (KB) medium. For isolation of fluorescent Pseudomonads, the method proposed by Vlassak, et al. [16] was followed with slight modification. Ten gram of soil sample was taken in a conical flask containing 100 ml of double-distilled water (ddW). The sample was agitated for 15 minutes on a vortex and allowed the soil particles to settle down. Serial dilution was made up to 10^{-8} . One millilitre of suspension from 10^{-6} dilution was used for isolation of fluorescent Pseudomonads by applying the pour plate method on King's-B (KB) medium.

The inoculated plates were wrapped with paraffin tape and incubated at room temperature ($28 \pm 2^\circ\text{C}$) for 24 h. Further, pure cultures of isolated colonies were obtained by the streak plate method. Fluorescent Pseudomonad colonies were identified under the UV light for their conformation and the cultures were named based on the location and the name of the crop from which they were collected (Table 1).

Morphological characteristics (colony morphology, colour, pigmentation at the lower surface of the Petri plate etc.) of each isolate was examined on King's-B medium by following serial dilution techniques (up to 10^{-8} dilution). The experiment was conducted in three replications and incubated at $28 \pm 2^\circ\text{C}$ for 24 h. Morphological diversity of isolates was observed by characteristics of colonies such as shape, margin, colour and pigmentation.

Physiological characterization of fluorescent Pseudomonads was studied by undertaking the following tests. Hydrogen ion concentration of King's B medium was adjusted with 0.1N alkali (NaOH) or acid (HCl) in Corning pH meter model No. 7 (Corning Scientific Instruments) by using disodium hydrogen phosphate citric acid buffer according to the schedule of Vogel [17]. Before autoclaving, the pH was adjusted to 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, and 8.0. Different fluorescent

Pseudomonads isolates were inoculated separately to different Petri plates by pour plate method using 10^{-8} dilution of pure culture suspension. Then media with different pH was poured on to the respective plates. Three replications were maintained and incubated at $28 \pm 2^\circ\text{C}$ for 24 h. After incubation, the number of colonies per plate was calculated.

Table 1. The naming of fluorescent pseudomonads isolates according to the place of collection

Sl. no.	Crop	Area	Abbreviation
1	Wheat	Sabour	WHSB
2	Linseed	Sabour	LNSB
3	Mustard	Sabour	MSSB
4	Chickpea	Sabour	CKSB
5	Bamboo	Sabour	BMSB
6	Tomato	Sabour	TMSB
7	Brinjal	Ghogha	BRGH
8	Chilli	Ghogha	CHGH
9	Bamboo	Ghogha	BMGH
10	Tomato	Ghogha	TMGH
11	Brinjal	Kahalgaoon	BRKH
12	Wheat	Kahalgaoon	WHKH
13	Brinjal	Nathnagar	BRNT
14	Mustard	Kishanganj	MSKSN
15	Maize	Kishanganj	MZKSN
16	Marigold	Kahalgaoon	MRKH

For studying the effect of carbon source on the growth of fluorescent pseudomonads, dextrose was replaced with glucose, fructose, sucrose and mannitol respectively, in nutrient agar medium. Different fluorescent Pseudomonads were inoculated to each carbon compound containing media separately by pour plate method. They were replicated thrice and incubated at $28 \pm 2^\circ\text{C}$ for 24 h. After incubation, the numbers of colonies were recorded. The method applied was a Completely Randomized Design (CRD).

3. RESULTS AND DISCUSSION

Sixteen fluorescent Pseudomonad cultures were isolated on King's-B medium and pure cultures of the isolates were maintained at the Department of Plant Pathology. Morphological studies revealed that maximum number (51.33) of colonies of fluorescent Pseudomonads were observed in isolate WHSB followed by isolate MSSB (28.33), BRKH (26.67), BMSB (26.33), TMSB (26.00), MZKSN (22.33), MSKSN (21.33), WHKH (20.00), CKSB (19.00) and TMGH (17.67), respectively. Whereas, least number of colonies were observed in BRGH (10.33)

Table 2. Morphological characteristics of fluorescent pseudomonad isolates on King's B medium

Sl. No.	Isolate	No. of colonies /plate (10^{-8})	Colony shape	Colony colour	Pigmentation at lower surface of plate
1	WHSB	51.33	Round	Yellowish white	Light green
2	LNSB	12.00	Irregular	Green	Light green
3	MSSB	28.33	Round	Dull white	Dark green
4	CKSB	19.00	Round	Dull white	Light green
5	BRGH	10.33	Irregular	Green	Dark green
6	BRKH	26.67	Round	Whitish green	Bluish white
7	CHGH	11.33	Round	Green	Dark green
8	WHKH	20.00	Round	Green	Dark green
9	BMSB	26.33	Round	Yellowish	Light green
10	BMGH	14.00	Round	Yellowish white	Light green
11	BRNT	11.33	Round	Green	Dark green
12	TMGH	17.67	Irregular	Green	Bluish-green
13	TMSB	26.00	Round	Yellowish green	Bluish-green
14	MSKSN	21.33	Round	Green	Dark green
15	MZKSN	22.33	Round	Green	Dark green
16	MRKH	12.67	Irregular	Whitish green	Light green
C.D		4.57			
SE(m)		1.58			

Table 3. Growth of fluorescent pseudomonads at different pH

Sl. No.	pH	Isolate 1(High fluorescent) MSSB	Isolate 2 (Low fluorescent) WHSB
		No. of colonies per plate	No. of colonies per plate
1	4.0	0.67	0.00
2	5.0	6.33	4.00
3	6.0	14.67	6.67
4	6.5	27.33	18.67
5	7.0	64.33	40.00
6	7.5	78.33	63.67
7	8.0	39.67	36.67
SE(m)		2.24	1.82
CD		6.86	5.57

Table 4. Growth of fluorescent pseudomonads on different carbon sources

Sl. No.	Carbon sources	Isolate 1(High fluorescent) MSSB	Isolate 2 (Low fluorescent) WHSB
		No. of colonies per plate	No. of colonies per plate
1	Glucose	61.00	44.33
2	Fructose	18.67	14.00
3	Sucrose	14.00	12.00
4	Dextrose	51.67	32.67
5	Mannitol	42.00	24.00
SE(m)		1.45	1.45
CD		4.63	4.57

followed by BRNT (11.33), CHGH (11.33), LNSB (12.00) and MRKH (12.67), respectively at 10^{-8} dilution (Table 2). The huge amount of variability in the population of fluorescent pseudomonads is an indication of the different levels adaptability of fluorescent pseudomonads to different crops.

Among sixteen isolates, twelve isolates were found producing round to oval-shaped colonies viz., WHSB, MSSB, CKSB, BRKH, CHGH, WHKH, BMSB, BMGH, BRNT, TMSB, MSKSN and four isolates produced irregular colonies viz., TMGH, MRKH, LNSB and BRGH on King's-B agar medium. Colony colour varied

from isolate to isolate viz., slightly yellowish-white, green, dull-white and whitish-green to yellowish. Fluorescent Pseudomonads produced different colour pigmentation (light green, dark green, bluish-white and bluish green) at the lower surface of King's-B medium (Table 1).

The levels of fluorescens among the isolates varied considerably indicating variability in pigment production ability. Both the selected isolates, high fluorescent isolate (MSSB) and low fluorescent isolate (WHSB) preferred pH 7.5 for their optimum growth followed by pH 7.0 and 8.0, respectively (Table 3). The same isolates also preferred glucose as a carbon source followed by dextrose for their rapid growth (Table 4). Similar results were observed with Wasi, et al. [18] who characterized fluorescent Pseudomonads based on morphological, cultural and biochemical properties and fluorescence activity, and presumptively identified as *Pseudomonas fluorescens*.

Rekha, et al. [19] isolated proteobacterium from rhizosphere soil and identified using morphological and cultural characteristics. Among 32 bacteria, 14 of them have been identified as fluorescent pseudomonads. Anitha and Kumudini (2014) also characterized *Pseudomonas* spp. on the bases morphological and physiological properties. Babu and Paramageetham [20] isolated a total of 10 strains of *P. fluorescens* from forest soils. Most of the isolated bacteria developed pale green to dark green pigmentation on the king's B agar medium.

Soesanto, et al. [21] worked on morphological features of *Pseudomonas fluorescens* P60 and identified the greenish-yellow fluorescent activity of *P. fluorescens* P60 under the sunlight. Meera and Balabaskar [22] isolated thirty-five of *Pseudomonas* spp. from the rhizosphere of rice fields, among these, seven isolates showed bright fluorescence under UV light. Soesanto, et al. [21] worked on physiological features of *Pseudomonas fluorescens* P60 based on the physiological characteristic; the bacteria had optimal pH 7-8 for their better growth.

Haggag and Soud [8] investigated the effects of fermentation parameters (pH, carbon and nitrogen concentration), and concluded that glycerol was found to be the best carbon source for improved biomass production. Similarly, Anitha and Kumudini (2014) also studied the utilization of sucrose, dextrose, mannitol and lactose by the *Pseudomonas* spp.

The isolates utilized sucrose, mannitol and lactose to a varying extent. The use of biological control agents as an alternative to fungicides is increasing rapidly in present-day agriculture due to the deleterious effects of chemical pesticides.

4. CONCLUSION

Fluorescent pseudomonads, a major constituent of rhizobacteria, encourage the plant growth through their diverse mechanisms. They are one among the PGPR known to inhibit plant pathogenic fungi by the production of secondary metabolites. The huge variability among the population of fluorescent pseudomonads opens scope for exploration of location-specific isolates suitable for different agro-ecologies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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