International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
www.chemijournal.com
IJCS 2020; 8(4): 2429-2433

Received: 08-05-2020
Accepted: 10-06-2020

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Collection, isolation and characterization of the *Pseudomonas fluorescence*, from Rhizosphere of Different crops (Ragi, Pigeonpea and groundnut)

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DOI: https://doi.org/10.22271/chemi.2020.v8.i4ab.9995

Abstract
Pseudomonas fluorescens is a common, nonpathogenic, gram-negative and rod-shaped, bacterium that colonize primarily in soil, plant and water. *Pseudomonas fluorescens* is one of the important group of bacteria which play a major role in the plant growth promotion, induced systemic resistance and also biological control of pathogens. The present study was conducted to isolate and characterization of *Pseudomonas fluorescens* from rhizosphere of different crops like Ragi, Pigeonpea and Groundnut. Twenty *Pseudomonas fluorescens* isolates were isolated on King's B Base medium from rhizosphere soil collected from chintamani, chikkaballapura district of karanataka. Based on morphological characterization and biochemical tests the isolate collected from rhizospere soil of groundnut were found to more prominant isolate compare to other isolates and these isolates were further confirmed by PCR using 16S rRNA and *Pseudomonas fluorescens* specific primers.

Keywords: Pseudomonas fluorescens, biocontrol. Rhizosphere, PGPR

Introduction
Biological control of plant pathogens by antagonistic microorganisms is a potential non-chemical means (Harman, 1991) [10] and is known to be a cheap and effective eco-friendly method for the management of crop diseases (Cook and Baker, 1983) [5]. The use of biological control agents as an alternative to fungicides is increasing rapidly in the present day agriculture due to the deleterious effects of chemical pesticides. Members of the genus Pseudomonas and Trichoderma have long been known for their potential to reduce the plant disease caused by fungal pathogens and they have gained considerable importance as potential antagonistic microorganisms (Pant and Mukhopadhyay, 2001) [18]. Among these the bacterial antagonists have the twin advantage of faster multiplication and higher rhizosphere competence hence, *P. fluorescens* have been successfully used for biological control of several plant pathogens (Ramamoorthy *et al*., 2002) [20] and biological control using PGPR strains especially from the genus Pseudomonas is an effective substitute for chemical pesticides to suppress plant diseases (Compant *et al*., 2005) [4].

Suppression of soil borne fungal pathogens by *P. fluorescens* isolates usually depends on the ability to produce antifungal metabolites (Keel *et al*. 1992; Dowling & O'Gara 1994; Thomashow & Weller 1996) [11, 7, 25]. The plant growth promotion by fluorescent Pseudomonads is by both direct and indirect mechanisms (Patten & Glick 1996). Direct growth promotion is due to production of phytohormones, solubilisation of phosphates (Katznelson & Bose 1959; Antoun & Kloepper 2001) [12, 1], increased uptake of iron through production of siderophores (Gupta *et al*. 2002; Chaiharn *et al*. 2009) [8, 3] and volatile metabolites. Indirect methods of growth promotion are due to antibiosis, Hydrogen cyanide (HCN) (Dowling & O'Gara 1994) [7], competition for space and nutrients, parasitism or lysis of pathogen hyphae, inhibition of pathogen-produced enzymes or toxins and through induced systemic resistance (Nanda Kumar *et al*. 2001) [16]. A successful agent should be able to prevent the proliferation and subsequent establishment of a soilborne pathogen from crop rhizosphere. Identification of elite Pseudomonads with abilities to produce metabolites and antibiotics is a key step in formulating effective disease management strategies. Extracellular

metabolites of *P. fluorescens* are potentially antagonistic to several plant pathogens (Hass & Defago 2005) [9]. Metabolites of *P. fluorescens* such as HCN, antibiotics (2, 4-diacetylphloroglucinol (2, 4-DAPG) and phenazine-1 carboxylic acid, enzymes, hormones, etc., play an important role in suppressing soilborne diseases of several crops (Sharma & Dubin 1996) [22]. therefore, in the present study we isolated and characterized *Pseudomonas fluorescens* from Rhizospheric soils of different crop plants Ragi, Pigeonpea and Groundnut in Karanataka.

Materials and Methods

Soil sample collection, isolation and purification of *P. fluorescens*

Soil samples were collected from the rhizosphere of different crops *viz*., Ragi, Pigeonpea and Groundnut from different locations of Chintamani, Chikkaballapur district of Karnataka. The soil thus obtained was mixed with 100ml sterile water and kept for shaking for 20-30 min to obtain standard soil suspension. Isolation of *P. fluorescens* was made by following the serial dilutions and pour plate method.

The selective and specific King's B media was used for isolation of *P. fluorescens*. One ml of soil suspension from aliquot dilutions (10^4 to 10^6) was aseptically added to sterile petri plates containing twenty ml of sterile medium and incubated at 28 ±20C for 48 h. after incubation well separated single colonies exhibiting greenish yellow fluorescens under UV-light (365nm) were picked and further purified on fresh King's B agar medium. The pure cultures obtained were stored in 50% glycerol at-80 °C for further use.

Morphological characterization

Pure cultures of the selected isolates were streaked on King's B agar Petri plates separately for colony development. The individual colonies were examined for shape, size, structure of colonies and pigmentation. The isolates were Gram stained by smearing loop full culture on clean slides and examined microscopically for shape, arrangement and Gram reaction.

Biochemical characterization

For the identification of *P. fluorescens*, certain biochemical tests were conducted

Gram staining

A loopful bacterial culture was transferred on a clean slide and a smear was made which was air dried and heat fixed. The smear was flooded for one min. with ammonium oxalate crystal violet. Excess strain was poured off and the slide was washed in a gentle stream of water. Lugol's iodine solution was applied and allowed to remain for one min. decolorized with 95 per cent ethyl alcohol. The smear was washed in gentle stream of water and counter stained with safranin for 30 seconds. The Gram negative cells appeared red in color and Gram positive cells appeared violet in color (Cyrabree and Hindshill, 1975) [6].

Phosphate Solubilization test

Phosphate solubilization ability of *Pseudomonas fluorescens* isolates were tested on Pikovaskya′s agar medium (Hi–Media Ltd., India) which contain insoluble dicalcium phosphate. One loopful of overnight grown cultures each isolate was spotted on Pikovaskya′s agar medium and incubted at 28°C for 24 hour and was observed for zone of clearance (Wahyudi *et al*., 2011) [27].

Fluorescent pigment production

Petri plates containing King's B agar media was inoculated with *P. fluorescens* isolates and incubated for 3-4 days for observation of pigment production. Yellowish green fluorescent pigment observed under UV light (365nm) indicated positive results.

Urea test

Petri dishes containing urea agar were inoculated with different *P. fluorescens* isolates and Petri plates were incubated at 28°C for 48 hours. Production of urease enzyme was observed (Sinha and Simon, 2013).

Protease test

To determine the protease production each *P. fluorescens* isolates were streaked on to Petri plates containing skim milk agar and incubated for three days at 28 °C.

Starch hydrolysis

Petri plates containing Nutrient agar supplemented with 0.5% of starch were streaked with *P. fluorescens* isolates collected from rhizospheric soils and incubated at 28 °C for three days. The bacterial growth was then inundated with Lugol solution (Soesanto *et al*., 2011) [23].

Gelatin hydrolysis

To determine gelatin hydrolysis, the overnight grown Pseudomonas isolates were inoculated in to Petri plates containing nutrient agar supplemented with gelatin and incubated at at 28°C for 24 hour and then plates were kept in refrigerator for 30 min at 4 °C. Gelatin hydrolysis was observed by liquefied media (Suman *et al*., 2015) [24].

Molecular characterization

Genomic DNA extraction and PCR: DNA was isolated and subjected to PCR analysis. Genomic DNA was extracted from bacterial cultures grown in Nutrient Broth. After 24 h, 50 mL of culture was removed and centrifuged at 3000 × g for 5 min, after which the cells were washed in 0.85% NaCl solution, re-centrifuged and resuspended in 2 mL of TE buffer (100 mM EDTA; 150mM NaCl; 100 mM Tris-HCl, pH = 8.0) containing 4 mg mL-1 lysozyme. The suspension was incubated at 37°C for 45 min and 0.5 mL of 8.5% SDS was added, followed by incubation at 75°C for 30 min before the addition of 1.5 mL of potassium acetate (5 M, pH=5.2) and incubated for 20 min at 4°C. The DNA was extracted with chloroform: isoamyl alcohol (24:1), precipitated with ice-cold isopropanol, washed with 70% ethanol, briefly dried and re-suspended in 200µL of TE buffer. The concentration of DNA was measured using a Biowave S2100 Diode Array spectrophotometer and stored at -20°C until further use.

Molecular identification of the bacterial isolates was based The 16S rRNA gene amplification by PCR using the following universal primer pair: 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Lane, 1991) [13]. *Pseudomonas fluorescens* specific gene encoding *cumin deoxygenase* (*cds*) *was am*plified using forward cds F: TTGAGCCCCGTTACATCTTC and reverse cdsR: GGGGAACCCACCTAGGATAA, which were developed from the *cumene dioxygenase* gene sequence in the GenBank accession no. D37828.1 (Mohammed A. H. 2015) [15]. The PCR amplification was carried out in 20 µl reaction mixtures that consisting 50 ng of DNA, 1X PCR buffer, 10 mM of dNTPs mixture, 10 pmol of each primer and 1 unit of Taq

DNA polymerase. The PCR program included initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 45s, 56°C for 45s, 72°C for 60s, and then a final extension at 72 °C for 10 min. (Ningaraju *et al*., 2018).

Result and Discussion

Twenty *Pseudomonas fluorescens* isolates were isolated on King's B Base medium from rhizosphere of different crops like Ragi, Pigeonpea and Groundnut. Soil samples were collected from chintamani, chikkaballapura district of karanataka for the isolation of native fluorescent pseudomonads. The samples were serially diluted and plated on to Petri plates containing King's B media. Colonies developed after 48 hours of incubation at 28 °C were purified onto fresh media and total twenty isolates were selected (table 1). All the isolates developed small to medium, smooth, glistening colonies, convex elevation and these isolates were Gram negative, rods without sporulation when observed under microscope. Out of the total 20 isolates, 5 isolates showed yellowish green pigmentation, 7 showed light green pigmentation and 8 showed bluish green pigmentation under UV light (table.2).

All the isolates showed, smooth shiny surface, Based on the colony morphology and cultural characteristics of the isolates on the KB medium and observation of pigmentation under UV light about twenty colonies from above plates were selected (Fig.1), purified and the pure cultures obtained was stored in refrigerator at 4º C (Fig.2). Similar results were obtained with Jayashree *et al*., 2000, who isolated fluorescent pseudomonads from the rhizospheres of blackgram, carrot, banana, pepper, rice and forest trees grown in several geographical areas of Tamil Nadu and later on confirmed the fluorescent colonies by viewing under UV-light.

Table 1: Source and identity of fluorescent *Pseudomonas* isolates

Rhizosphere of crop	Designation of isolates	Number of isolates
Ragi	RPF	5
Pigeon pea	PPPF	7
Groundnut	GNPF	8

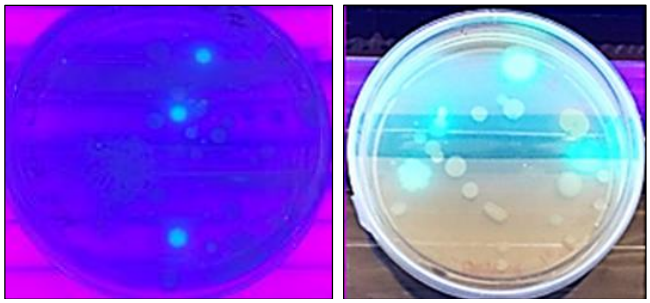

Fig 1: Identification and confirmation of Pseudomonas Fluorescens grown on king's B base agar media under UV light

Table 2: Morphological characteristics of *P. fluorescence* isolates

Isolate	Cell shape	Colony type	Colony color and Emission of fluorescens to UV light	Growth type
RPF1	Rod	Round	Yellowish green	Slow
RPF2	Rod	Round	Yellowish green	Slow
RPF3	Rod	Round	Yellowish green	Slow
RPF4	Rod	Round	Yellowish green	Slow
RPF5	Rod	Round	Yellowish green	Slow
PPPF1	Rod	Convex	Light Green	Fast
PPPF2	Rod	Convex	Light Green	Fast
PPPF3	Rod	Convex	Light Green	Fast
PPPF4	Rod	Convex	Light Green	Fast
PPPF5	Rod	Convex	Light Green	Fast
PPPF6	Rod	Convex	Light Green	Fast
PPPF7	Rod	Convex	Light Green	Fast
GNPF1	Rod	Round	Bluish green	Fast
GNPF2	Rod	Round	Bluish green	Fast
GNPF3	Rod	Round	Bluish green	Fast
GNPF4	Rod	Round	Bluish green	Fast
GNPF5	Rod	Round	Bluish green	Fast
GNPF6	Rod	Round	Bluish green	Fast
GNPF7	Rod	Round	Bluish green	Fast
GNPF8	Rod	Round	Bluish green	Fast

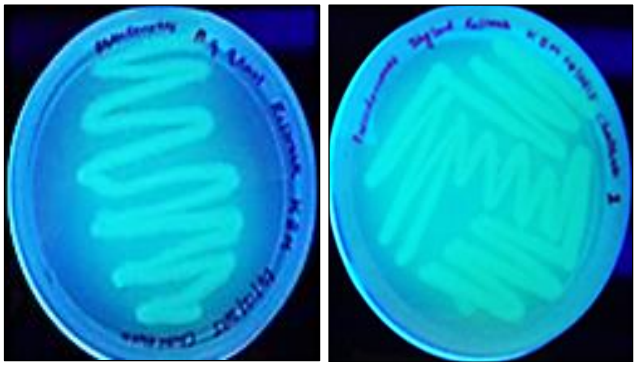

Fig 2: Purification of isolated Pseudomonas Fluorescens isolates King's B base media

Biochemical Characterization

Different biochemical tests revealed that all the twenty isolates of *P. fluorescence* were positive for phosphate solubilization, urease production, protease enzymatic activity, starch hydrolysis and gelatin liquefaction (table 3). Pseudomonas isolates from groundnut rhizosphere alone showed urease activity and not observed in other isolates (figure 3). Similar results were obtained by T Meera and P Balabaskar 2012 [14], who isolated and characterized Pseudomonas *fluorescens* from rice fields. Biyyani Suman *et al*., 2015 [24] isolated the thirty native *P. fluorescens* isolates from the rhizosphere of rice in the Rangareddy district, Telangana and characterized by morphological, cultural and biochemical tests.

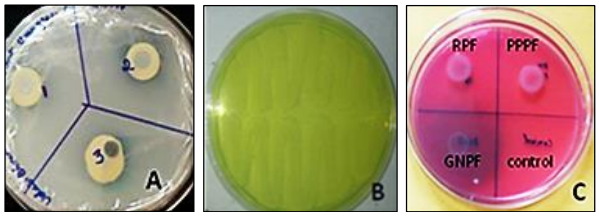

Fig 3: Representative Images of biochemical tests of pseudomonas Fluorescens isolates
A) Phosphate solubilization **B)** Pigment production **C)** Urease production

Table 3: Biochemical characteristics of *P. fluorescence* isolates

Sl No.	Character	*Pseudomonas fluorescens* isolates		
		RPF1-5	PPPF1-7	GNPF1-8
1	Grams reaction	Negative	Negative	Negative
2	Pigment production	Positive	Positive	Positive
3	Catalase test	Positive	Positive	Positive
4	Proteolytic activity	Positive	Positive	Positive
5	Starch hydrolysis	Positive	Positive	Positive
6	Gelatin liquefaction	Positive	Positive	Positive
7	Phosphate solubilization	Positive	Positive	Positive
8	Urease activity	Negative	Negative	Positive

Molecular characterization of *Pseudomonas fluoresces* isolates By PCR with bacterial specific primers (16s rRNA primers), 500bp amplification confirms the bacterial isolates (Fig. 4). Scarpellini *et al*. (2004) [21] first developed a 16S rRNA PCR-based assay to identify *P. fluorescens* isolates and their corresponding biotype. The confirmed bacterial isolates were further confirmed by *Pseudomonas fluoresces* specific *cumin deoxygenase* (*cds*) gene amplification with PCR by cds specific primers. Agarose gel electrophoresis showed nearly 500bp band, indicate the amplification of cds gene which confirms that these bacterial isolates ware *Pseudomonas fluoresces* only (Fig.5). Cumene deoxygenase (Cds) gene primers were used for PCR amplification which is particular for *Pseudomonas fluorescens* and not for other Pseudomonas spp. (Mohammed. A.H. 2015) [15].

Conclusion

Due to failure of many pesticides in plant disease control, secondary metabolites of biocontrol agents, such as fluorescent pseudomonads, were future ecofriendly weapons in targeting the plant diseases due to antimicrobial characteristics of those metabolites. Therefore, identifying native isolates of fluorescent pseudomonads were done from rhizosphere of different crops, among that *P. fluorescens* isolate from groundnut rhizosphere found to be more prominent in morphological and biochemical features as compare to other isolates in the present investigations. The results presented here provide an accurate, easy to perform and cheap method for the initial profiling of isolates belonging to the *P. fluorescens* complex of species isolated from Rhizosphere.

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