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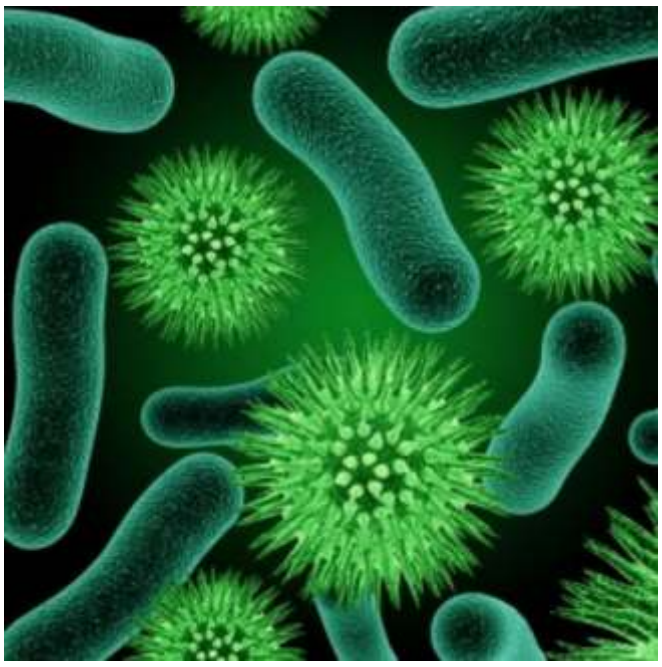
ISOLATION AND CHARACTERIZATION OF PHOSPHOBACTERIAL ISOLATES FROM BHENDI RHIZOSPHERE SOILS OF CUDDALORE DISTRICT



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Abstract:-In the present study, an attempt was made to isolate and characterize the phosphobacterial isolates such as *Bacillus sp.* and *Pseudomonas sp*. from fifteen bhendi rhizosphere soil samples collected from Cuddalore district of Tamil Nadu. The results showed that all rhizosphere soils harbored phosphobacterial isolates such as *Bacillus* and *Pseudomonas* to the tune of 104 CFU g^{-1} of rhizosphere soil sample. Pure culture of the above phosphobacterial isolates were prepared and further subjected to different characterization studies for their tentative identification. General and biochemical characterization studies clearly revealed that seven isolates of *B. megaterium*, three each isolates of *B. subtilis* and *B. polymyxa* and two isolates of B. cereus were found among the fifteen Bacillus rhizosphere soils. With regard to Pseudomonas population eight isolates of *P. fluorescens*, four isolates of *P. putida* and three isolates of *P. striata* were also obtained among the fifteen rhizosphere soils.

Keywords: Phosphobacteria, Bhendi, Rhizosphere, *Bacillus, Pseudomonas.*

INTRODUCTION

Bhendi is one of the most important vegetable crops in India due to its nutritional values like high amount of vitamin A, B and C, carbohydrates, phosphorus, magnesium and potassium. It is also a potential oil and protein crop which also has an exporting value accounting for thirteen per cent among fresh vegetables (Bose and Ranjan, 1988).

India is an agriculture based country, farmers need adequate resources to replenish soil fertility and maintain the productivity of soil. Really, the green revolution has popularized the use of chemical fertilizers to achieve higher productivity. But due to continuous and indiscriminate use of fertilizers, the natural fertility of soil has been lost and this activity has contaminated our soil, water and food. Hence, the farmers are in need of searching alternative to replace the chemical fertilizers. In recent days, the use of organic inputs like vermicompost, bio fertilizers and bio pesticides is becoming popular in the world wide in sustainable agriculture.

Phosphorous is the second most important macronutrient for growth and productivity of crop plants. It plays major role in plant cell division, photosynthesis and development of good root system and utilization of carbohydrates. Its deficiency results in the leaves turning brown accompanied by small leaves, weak stem and slow development. In ancient times the use of animal manures to provide phosphorous for plant growth was a common agricultural practice. Organically bound phosphorous enters in soil during the decay of natural vegetation, dead animals and from animal excretions. At that time, role of micro flora on soil fertility was hardly understood (Kannaiyan *et al*., 2004).

Phosphate solubilizing bacteria are very much important in crop production as they solubilize the insoluble forms of phosphates and uptake of other nutrients (Antoun *et a*l., 1998; Sperber, 1958; Cao *et al*., 1999; Khan *et al*., 2007) and also stimulates plant growth by providing hormones, vitamins and other plant promoting substances.

The use of phosphate solubilizing bacteria as inoculants simultaneously increases phosphate uptake by the plant and crop yield. Strains from the genera such as *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizing agents. The principal mechanism for mineral phosphate solubilisation is the production of organic acids and phosphatase enzymes play a major role in the mineralization of organic phosphorous in soil (Rodriguez *et al.*, 1999).

The role of microorganisms in solubilizing insoluble phosphate in soil and making it available to plants is well known (Kundu and Gaur, 1984). Phosphate solubilizing microorganisms include several genera of bacteria *viz.*, *Bacillus*, *Pseudomonas*, *Klebsiella* and *Serratia* (Nakas *et al.*, 1987; Strom and Lory, 1987; Dave and Patel, 1999). Among bacteria, most efficient phosphate solubilizing bacteria belonged to the genera *Bacillus* and *Pseudomonas* (Dave and Patel, 1999). The phosphate solubilizing bacterial isolates were screened based on the ability to release soluble phosphorous from apatite in the culture medium (Banik and Dey, 1982; White Law *et al*., 1999).

Several species of *Pseudomonas* are well known for aggressive root colonization, possessing plant growth promoting activities and potential bio control abilities have been well documented (Schroth and Hancock, 1982; Fravel, 1988; Weller, 1988; Homma *et al*., 1989; Hebbar *et al*., 1992; Jayaswal *et al*., 1993; Pandey *et al*., 1999).

Diverse reports have described Bacillus spp. and other gram-positive bacteria as an important group in solubilization and mineralization of P in aquatic and terrestrial environments (Puente *et al.*, 2004; Hill *et al.*, 2007).

Several strains of the species *Bacillus amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumillus*, *B. mycoides* and *B. sphaericus* elicit significant reductions in the incidence or severity of various diseases on a diversity of hosts was explained by Devendra Choudhary and Bhardish Jhori (2009). Kumar and Dube (1993) obtained strains of *P. putida* from tomato root using modified King's 'B' medium incorporated with cyclohexamide, ampicillin, chloramphenicol and pentachloro benzene. Benchabane (2004) isolated several fluorescent strains of *Pseudomonas* from the rhizosphere of different plants namely tomato, potato, corn and vine and suggested that the plant and the soil type play a considerable role in the distribution and the taxonomic diversity of fluorescent Pseudomonas. In the present investigation an attempt was made to isolate and characterize certain phosphobacterial isolates *viz.*, *Bacillus* and *Pseudomonas* from Bhendi rhizosphere soil samples of Cuddalore District, Tamil nadu, India.

MATERIALS AND METHODS

About fifteen locations from Cuddalore District were randomly selected and the Bhendi rhizosphere soil samples were collected and analyzed for their physico-chemical and microbiological properties by adopting the following protocols.

S.NO.	PARAMETER		REFERENCE
1	Soil pH	:	Erhart and Burian, 1997
2	Soil EC	:	Falcon *et al.*, 1987
3	Organic carbon	:	Jackson, 1973
4	Available nitrogen	:	Subbiah and Asija, 1956
5	Available phosphorus	:	Olsen *et al.*, 1954
6	Available potassium	:	Jackson, 1973
7	Enumeration of total heterotrophs	:	Allen, 1953; Johnson *et al.*, 1956
	Enumeration of Phosphobacteria isolates		
8.	*Bacillus*	:	Sperber, 1958
9.	*Pseudomonas*	:	King's *et al.*(1954)

Further the *Bacillus* and *Pseudomonas* isolates were subjected to biochemical and characterization studies by adopting the following standard protocols.

	Characterization of Phosphobacteria isolates		
1.	Gram's staining	:	Rangaswami, 1975
2.	Motility	:	Cheesbrough, 1984
3.	Spore staining	:	Hariigan and McCance, 1966
4.	Acid production	:	Krieg and Tarrand, 1978
5.	Hydrolysis of starch	:	Stolpe and Godkeri, 1981
6.	Hydrolysis of gelatin	:	Stolpe and Godkeri, 1981
7.	Hydrolysis of casein	:	Smibert and Krieg, 1981
8.	Catalase test	:	Aneja, 1993
9.	Oxidase test	:	Bergey's manuals of determinative Bacteriology 6th Edition
10.	Indole test	:	Seeley and Vandemark, 1981
11.	Methyl red test	:	Seeley and Vandemark, 1981
12.	Urease test	:	Christensen, 1946
13.	Voges-proskauer test	:	Aneja, 1993
14.	Citrate utilization test	:	Seeley and Vandemark, 1981

RESULT AND DISCUSSION

The physico-chemical properties and nutrient status of the fifteen bhendi rhizosphere soil samples of Cuddalore distract were analyzed and presented in (Table - 1). The pH (soil reaction) of the soil samples ranged between 7.03 and 7.37 and electrical conductivity (EC) between 1.14 and 1.82 dSm^{-1} levels. With regard to nutrient contents, the available nitrogen content ranged from 131.42 to 173.46 kg ha^{-1}, available phosphorus ranged from 17.34 to 31.23 kg ha^{-1} and available potassium from 235.62 to 290.75 kg ha^{-1} were recorded. The higher values of available nitrogen (173.46 kg ha^{-1}), phosphorus (31.23 kg ha^{-1}) and potassium (290.75 kg ha^{-1}) were recorded in Sakkangudi soil samples. The vital nutrient parameter, organic carbon content of the bhendi rhizosphere soil samples were ranged between 0.55 and 0.98 per cent ranges from Sakkangudi and Keerapalaiyam respectively. All other soil samples OC content were found in between these values. It was well understood that these physico-chemical and nutritional parameters are of heterogeneous nature that are determined by different environmental and cultural practices.

The bhendi rhizosphere soil samples were subjected to enumeration of total heterotrophic microbial population viz., bacteria, fungi and actinobacteria with respective agar medium by employing standard plate count method and the results are presented in (Table - 2). The results revealed that the population load of 10^6, 10^5 and 10^4 CFU g^{-1} of soil levels were observed for bacteria, fungi and actinobacteria respectively. The higher bacterial population of 48.33×10^6, fungal population of 10.67×10^5 and actinobacteria population of 10.00×10^4 CFU g^{-1} of soil were observed in Sakkangudi soil. The variation between the population levels of total heterotrophs may be due to different location, environment and cultural factors.

In addition to enumeration of total heterotrophic microbial population, the bhendi rhizosphere soil samples were further determined for the population of important phosphobacterial isolates like *Bacillus* and *Pseudomonas* (Fig. 1). The results revealed that all the bhendi rhizosphere soils harbored phosphobacteria to the tune of 10^4 CFU g^{-1} of oven dry soil. The higher Bacillus population of 12.67×10^4 CFU g^{-1} and minimum population of 5.67×10^4 CFU g^{-1} were recorded in Sakkangudi and Keerapalaiyam locations respectively. The maximum Pseudomonas of 9.33×10^4 CFU g^{-1} of oven dry soil at Sakkangudi location followed by 9.00×10^4 CFU g^{-1} oven dry soil (Orathur) and 8.67×10^4 CFU g^{-1} oven dry soil (Kattumanarkovil) and least population of 4.00×10^4 CFU g^{-1} of oven dry soil (Keerapalaiyam) soil sample were recorded. The phosphobacterial isolates obtained from the fifteen different locations were designated as BRB-1 to BRB-15 for Bacillus and BRP-1 to BRP-15 for *Pseudomonas* isolates.

The results showed that there are some variations among the population of these PGPR and marked reduction than the total heterotrophic bacteria. The relative occurrences of these phosphobacterial isolates against total heterotrophs were reported earlier by many researchers in maize and other crops (Balandreau *et al*., 1975; Baldani and Dobereiner, 1980; Guemouri - Athmani *et al*., 2000).

The colonization of *Pseudomonas* spp. in many important crops have been already many authors (Brown, 1974; Suslow and Schroth, 1982; Schroth and Hancock, 1982; Curl and Truelove, 1985; Fravel, 1988; Weller, 1988; Homma *et al*., 1989; Chanway *et al*., 1989; Hebbar *et al*., 1992; Jayaswal *et al*., 1993; Bakker *et al*., 1996; Pandey *et al*., 1998; 1999; Anita Pandey *et al*., 1999).

Jorquera *et al*. (2008) isolated *Phosphobacteria* [phytate-mineralizing bacteria (PMB) and phosphate-solubilizing bacteria (PSB)] from the rhizosphere of perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*), wheat (*Triticum aestivum*), oat (*Avena sativa*), and yellow lupin (*Lupinus luteus*) growing in volcanic soils of Chile which contains large amounts of total and organic phosphorus but low available phosphorus.

Hui *et al*. (2011) isolated about 74 strains of phosphate solubilizing bacteria (PSB) and 138 strains of phosphorus mineralizing bacteria (PMB) from 797 colonies and finally selected 3 PSB strains (*Pseudomonas fluorescens*) with highest Phosphorus solubilizing efficiency and 2 highest Phosphorus mineralizing strains (*Bacillus cerus* and *Bacillus subtilis*) for pot culture and filed hadien on population.

Adhkari *et al*. (2013) isolated seventy phosphobacterial isolates from rhizosphere soils like bhendi, chilli groundnut, brinjal, cabbage and tomato from different agro ecological regions of West Bengal and found twenty one *P. fluorescens* (biovar I, II, III) had antagonistic activity against *Rhizoctonia solani* under *in vitro* conditions.

Presence of Phosphobacterial isolates *viz., Bacillus* and *Pseudomonas* from bhendi rhizosphere soils of different regions were already documented by (Sivakumar and Tholkappian, 2013).

GENERAL AND BIOCHEMICAL CHARACTERIZATION OF *BACILLUS* ISOLATES

The *Bacillus* isolates obtained from fifteen bhendi rhizosphere soil samples were studied with general and biochemical characters (Table - 3). The results clearly revealed that they were Gram positive, motile, spore forming and acid producing *Bacilli*. Further they were positive for starch and gelatin hydrolysis, negative for casein hydrolysis, positive for catalase, oxidase and methyl red test, negative for urease test and produced mixed kind of results for indole test, Voges-Proskauer and citrate utilization tests. The results showed that the isolates BRB-1, BRB-2, BRB-3, BRB-7, BRB-8, BRB-11 and BRB-12 were *B. megaterium* and BRB- 4, BRB-6 and BRB-10 were *B. subtilis* and BRB-5, BRB-9 and BRB-15 were *B. polymyxa* and the remaining isolates *viz*., BRB-13 and BRB-14 were found to be *B. cereus*.

GENERAL AND BIOCHEMICAL CHARACTERIZATION OF *PSEUDOMONAS* ISOLATES

All the *Pseudomonas* isolates showed Gram negative, rod shaped motile cells during microscopic observation. Further they positively responded for pigmentation in King's B agar medium that confirmed their identity. Different biochemical characterization studies revealed that the isolates BRP-1, BRP-2, BRP-3, BRP-5, BRP-9, BRP-11, BRP-13 and BRP-14 were tentatively identified as *P. fluorescens*, BRP-4, BRP-6, BRP-10 and BRP-12 were identified as *P. putida* and the remaining isolates namely BRP-7, BRP-8 and BRP-15 were identified as *P. striata* (Table - 4).

CONCLUSION

The present study concluded that Bhendi rhizosphere soil samples collected from fifteen different locations of Cuddalore District of Tamil Nadu harbored its own native phosphobacteria *viz.*, *Bacillus* and *Pseudomonas* to the tune of 10^4 CFU g^{-1} levels on oven dry basis and their species level tentative identification was done based on general and certain biochemical characteristics.

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Table - 1: Physico-chemical properties of Bhendi rhizosphere soil samples of Cuddalore District

Table - 2: Determination of total heterotrophic microbial populations from Bhendi rhizosphere soil samples of Cuddalore District

S. No.	Location	Microbial population (CFU g^{-1} of soil)*		
		Bacteria ($\times 10^6$)	Fungi ($\times 10^4$)	Actinobacteria ($\times 10^4$)
1	Annamalai Nagar	19.67	7.67	7.11
2	Sakkangudi	12.11	10.67	10.00
3	Puliyankudi	11.67	9.11	8.67
4	Keerapalayam	11.00	4.67	4.00
5	Dhathampettai	15.11	6.00	5.67
6	Ayyipettai	17.00	6.67	6.11
7	Orathur	12.67	10.11	9.67
8	Sethiyathoppu	11.00	8.11	8.00
9	Bhuvanagiri	18.11	7.11	7.00
10	Perumathur	12.11	8.67	8.11
11	Kattumannarkovil	12.00	10.00	9.11
12	Kannangudi	11.67	5.67	5.11
13	Virudhachalam	11.11	5.11	4.67
14	Allapakkam	17.67	7.00	6.67
15	Melaparthikudi	16.00	6.11	6.00

* - Population on oven dry basis

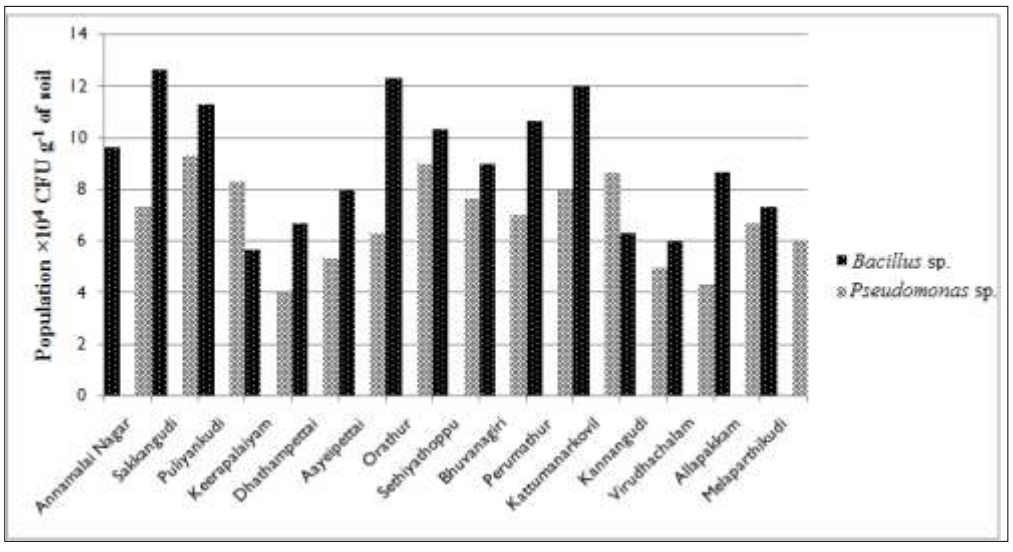

Fig. 1- Determination of certain phosphobacteria population from Bhendi rhizosphere soil samples of Cuddalore District

Table - 3: Characterization of *Bacillus* isolates obtained from the Bhendi rhizosphere soil samples of Cuddalore District

Isolates Code	Gram reaction	Motility	Spore staining	Acid production	Hydrolysis of starch	Hydrolysis of gelatin	Hydrolysis of Casein	Catalase test	Oxidase test	Urease test	Indole test	Methyl red test	Voges- Proskauer test	Utilization of citrate	Tentative Identification
BRB-1	+	+	+	+	+	+	-	+	+	-	+	+	-	+	*B. megaterium*
BRB-2	+	+	+	+	+	+	-	+	+	-	+	+	-	+	*B. megaterium*
BRB-3	+	+	+	+	+	+	-	+	+	-	+	+	-	+	*B. megaterium*
BRB-4	+	+	+	+	+	+	-	+	+	-	-	+	+	+	*B. subtilis*
BRB-5	+	+	+	+	+	+	-	+	+	-	-	+	+	-	*B. polymyxa*
BRB-6	+	+	+	+	+	+	-	+	+	-	-	+	+	+	*B. subtilis*
BRB-7	+	+	+	+	+	+	-	+	+	-	+	+	-	+	*B. megaterium*
BRB-8	+	+	+	+	+	+	-	+	+	-	+	+	-	+	*B. megaterium*
BRB-9	+	+	+	+	+	+	-	+	+	-	-	+	+	-	*B. polymyxa*
BRB-10	+	+	+	+	+	+	-	+	+	-	-	+	+	+	*B. subtilis*
BRB-11	+	+	+	+	+	+	-	+	+	-	+	+	-	+	*B. megaterium*
BRB-12	+	+	+	+	+	+	-	+	+	-	+	+	-	+	*B. megaterium*
BRB-13	+	+	+	+	+	+	-	+	+	-	-	+	-	-	*B. cereus*
BRB-14	+	+	+	+	+	+	-	+	+	-	-	+	-	-	*B. cereus*
BRB-15	+	+	+	+	+	+	-	+	+	-	-	+	+	-	*B. polymyxa*

(+) Showed positive growth; (-) Showed negative growth

Table - 4: Characterization of *Pseudomonas* isolates obtained from the Bhendi rhizosphere soil samples of Cuddalore District

Isolates code	Gram reaction	Motility	Starch hydrolysis	Hydrolysis of gelatin	Egg yolk reaction	Pigment production	Casein Hydrolysis	Catalase test	Oxidase test	Indole test	Methyl test	Citrate utilization test	H_2S Production	Nitrate reduction	Tentative Identification
BRP-1	-	+	-	+	-	+	+	+	+	-	-	+	-	-	*P. fluorescens*
BRP-2	-	+	-	+	-	+	+	+	+	-	-	+	-	-	*P. fluorescens*
BRP-3	-	+	-	+	-	+	+	+	+	-	-	+	-	-	*P. fluorescens*
BRP-4	-	+	-	-	+	+	+	+	+	-	-	+	-	-	*P. putida*
BRP-5	-	+	-	+	-	+	+	+	+	-	-	+	-	-	*P. fluorescens*
BRP-6	-	+	-	-	+	+	+	+	+	-	-	+	-	-	*P. putida*
BRP-7	-	+	-	-	+	+	+	+	+	-	-	+	-	-	*P. striata*
BRP-8	-	+	-	-	+	+	+	+	+	-	-	+	-	-	*P. striata*
BRP-9	-	+	-	+	-	+	+	+	+	-	-	+	-	-	*P. fluorescens*
BRP-10	-	+	-	-	+	+	+	+	+	-	-	+	-	-	*P. putida*
BRP-11	-	+	-	+	-	+	+	+	+	-	-	+	-	-	*P. fluorescens*
BRP-12	-	+	-	-	+	+	+	+	+	-	-	+	-	-	*P. putida*
BRP-13	-	+	-	+	-	+	+	+	+	-	-	+	-	-	*P. fluorescens*
BRP-14	-	+	-	+	-	+	+	+	+	-	-	+	-	-	*P. fluorescens*
BRP-15	-	+	-	-	+	+	+	+	+	-	-	+	-	-	*P. striata*

(+) Showed positive growth; (-) Showed negative growth