

Original Research Article

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Enumeration, Isolation and Identification of Diazotrophic Bacteria in Rhizosphere Soil of Two Rice Varieties in Jammu District, India

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ABSTRACT

Free living diazotrophic bacteria were isolated from the rhizosphere soil of two cultivated rice varieties grown in R. S. Pura and Bishnah blocks of Jammu district of Jammu Division of J&K State. Soil samples were collected from rice rhizosphere at panicle initiation stage. A total of twenty rice grown villages were selected and from each village two composite samples were taken from five plants one each from variety Basmati-370 and Ratna through random sampling from each rice growing village of R. S. Pura and Bishnah blocks of Jammu district by using GPS. The bacterial isolates characterized by various biochemical tests performed on them were identified by characteristics as outlined in Bergeys Manual of Systematic Bacteriology. The physico chemical properties of the soil were also estimated and correlated with changes in different environmental factors such as soil moisture, pH, EC, OC, N, P, K contents in different areas of R. S. Pura and Bishnah blocks and found to have influenced nitrogen fixation in soil thus affecting nitrogen fixers in the soil. The study highlighted the aerobic and anaerobic nature of isolates based on biochemical tests viz. catalase, nitrate reduction, citrate utilization and oxidase tests performed on them.

Keywords

Enumeration, Nitrogen fixers, Biochemical tests, *Azotobacter*, *Beijerinckia*, Characterization, Identification

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Introduction

Rice is the agricultural crop with the third-highest worldwide production after sugarcane and maize (FAO, 2017). As a staple food it is the most widely consumed cereal by a large part of the world's human population, especially in Asia. Nitrogen is major nutrient limiting the high yield potential of modern rice cultivars. Due to the realization of importance of nitrogen in rice farmers have preferred N-fertilizer responsive varieties of rice. Therefore as an alternative to inorganic

nitrogen fertilizers free living nitrogen fixing bacteria have been considered for promoting plant growth (Ladha and Reddy, 2000; Park *et al.*, 2004). The demand of nitrogen can therefore be overcome partially by isolating beneficial bacteria due to their potential as biofertilizers (Vessey, 2003).

Basmati-370 and ratna rice varieties are cultivated mainly in two respective blocks of R. S. Pura and Bishnah in Jammu district of J&K. Nitrogen fixers could be an important component of sustainable agriculture systems.

The aim of the present research was to isolate, and identify diazotrophic bacteria (free living nitrogen fixers) in rice rhizosphere soil so that they can be characterized for nitrogen fixation to reduce the use of chemical nitrogenous fertilizers.

Materials and Methods

Soil sampling

Soil samples were collected from rice rhizosphere at panicle initiation stage. A total of twenty rice grown villages were selected and from each village two composite samples were taken from five plants one each from variety *Basmati-370* and *Ratna* through random sampling from each rice growing village of R.S. Pura and Bishnah blocks of Jammu district by using GPS (Table 1).

A total of twenty rice grown villages were selected and from each village two samples were taken, one from *Basmati-370* and other from variety *Ratna* through random sampling. The location of each village is given in Table 1.

Dilution of soil samples

The sampled rhizosphere soil from each site was mixed thoroughly to make a composite soil sample. Ten grams of each soil sample was then diluted to 100 ml to make 10^{-1} dilution and further serial dilutions were prepared to 10^{-8} dilution under aseptic condition.

Bacterial counts

The calculation for the total number of bacteria was done by plating soil dilution on nutrient agar medium and total number of nitrogen-fixers was counted by plating soil dilutions on nitrifying medium using plating enrichment and pour plate technique and

incubating the plates for 7-10 days at 30°C. The total count of the microorganisms was obtained by multiplying the number of cells per plate by the dilution factor

Bacteria/gm soil = Number of bacteria/ Wt. of soil x Dilution

Isolation of diazotrophic bacteria

Nitrogen fixers viz. *Azotobacter* and *Beijerinckia* (Becking, 1981) were isolated on selective medium viz., the total nitrifying population was quantified in *Basmati-370* and *Ratna* variety in rhizosphere soil of rice grown in different areas of Jammu district using nitrifying medium (Lewis and Pramer, 1958)

Microscopic examination and identification of gram staining

Bacteria was studied for colony and cell morphology following microscopic examination and further identification of gram staining (Gram, 1884).

Biochemical characterization of isolates

The bacterial isolates were characterized using various biochemical test performed viz. Catalase test, Citrate utilization test, Nitrate reduction test and oxidase test (Cappucino and Sherman, 1992).

Identification

The bacterial isolates characterized by various biochemical tests performed on them were identified by characteristics as outlined in Bergey's Manual of Systematic Bacteriology (vol. 2 revised edition).

Soil physico-chemical properties (Table 2)

The physico-chemical properties were analyzed as per the procedures outlined below:

pH

pH of the soil samples was determined in 1:2.5 soil: water ratio (w/v) with the help of glass electrode pH meter (Jackson, 1973).

Electrical conductivity

Electrical conductivity was estimated in 1:2.5 soil: water suspension with EC meter as given by Jackson (1973).

Organic carbon

Organic carbon was analyzed with the help of Rapid titration method as proposed by Walkley and Black (1934).

Moisture

Moisture content was determined by gravimetric method as given by Black C. A. (1965).

Texture

Textural class was determined by Hydrometer method as given by Piper (1966).

Nutrient status of the soil samples

The nutrient status of the soil samples were analyzed as per the following method:

Available nitrogen

Nitrogen was determined by using alkaline permanganate as per the modified Kjeldahl method proposed by Subbiah and Asija (1956).

Available phosphorus

The available phosphorus was determined by the method mentioned by Olsen *et al.*, (1954).

Available potassium

1N ammonium acetate was used as extractant and the available potassium content was determined by feeding the extract to flame photometer (Jackson, 1973).

Results and Discussion

Enumeration of total biomass and N-fixers in Jammu district

Our study revealed that total number of N-fixers and percentage of N-fixers using nitrifying medium was more in R. S. Pura as compared to Bishnah block (Table 2).

Bacterial morphology of Diazotrophic bacterial isolates in Jammu district

The appearance of the colonies of *Azotobacter* bacteria grown on selective medium were circular, raised in elevation, small and pin type in size and creamy white in colour. *Beijerinckia* colonies on selective medium were irregular, flat in elevation and small in size and white in colour. Bacterial colonies raised on nutrient agar medium were pinpoint in size, circular in elevation, round and off-white in colour (Table 3) these result are in conformity with those of Khan *et al.*, (2008)

Biochemical characterization of bacterial isolates in Jammu district

Gram negative bacteria were more predominant than gram positive in the rhizosphere soil of Basmati-370 and Ratna rice. In our study, majority of bacteria were gram negative which suggests that *Azotobacter* and *Pseudomonas* may be predominant in the rhizosphere. These results are in agreement with Bowen and Foster, (1978) and Liliñares *et al.*, (1994) who stated that the abundance of *Azotobacter* and *Pseudomonas* may be due to the existence of

more favorable environmental conditions for their growth and their ability to efficiently use nutrients provided to plants in the form of exudates and produce substances that inhibit the growth of other microorganisms in their vicinity.

All diazotrophic bacterial isolates (*Azotobacter*) obtained from rhizosphere soil of Basmati-370 (Table 4) and Ratna (Table 6) rice in RS Pura and Bishnah block of Jammu district were rod shaped. The maximum number of diazotrophic bacterial isolates (*Beijerinckia*) was rod shaped in Basmati-370 rhizosphere (Table 5).

The maximum no. of diazotrophic bacterial isolates (*Beijerinckia*) in Ratna rhizosphere (Table 7) were rod shaped followed by rod shaped and mature dumbbell shaped cells in soil sample number twelve, thirteen, fifteen and seventeen in Bishnah block. Similar results have been reported by Khan *et al.*, (2008).

Biochemical tests (Catalase, Nitrate reduction, Citrate utilization and Oxidase test) performed on bacterial isolates of diazotrophic bacteria obtained from rhizosphere soil of Basmati-370 and Ratna rice growing areas of RS Pura and Bishnah blocks of Jammu district showed positive results except few, which showed negative results. Catalase test were positive in majority of soil samples tested for diazotrophic bacteria.

Catalase production and activity was detected by adding substrate H₂O₂ to bacterial isolates. Organisms which produce enzyme breakdown the H₂O₂ and resulting oxygen production produces bubbles in the reagent drop, indicates positive test. Organisms lacking the cytochrome system also lack the catalase enzyme and were unable to breakdown H₂O₂ into H₂O and O₂ and were catalase negative. The enzyme was present in most cytochrome

containing aerobic and facultative anaerobic bacteria. In the absence of enzyme, the toxic concentration cannot be degraded when these organisms are cultivated in the presence of oxygen (Srivastava, 2013).

In case of nitrate reduction test, in anaerobic conditions bacteria used NO₃⁻ as a source of oxygen and reduced to nitrite i.e. nitrate reduction was positive. Our results are in line with the results of Karpagam and Nagalaxmi, (2014). The nitrate reduction confirms that the isolates could reduce nitrate to nitrite but not to nitrogen gas, consistent with their ability to fix nitrogen.

This suggests they are capable of breaking down nitrate containing fertilizers in case of anaerobic conditions in soil. Most bacteria utilize the available oxygen in the medium for their growth and rapidly produced anaerobic conditions for further reaction suggesting that aerobic condition also do prevail in soil sample tested.

Bacteria used citrate as sole source of carbon and nitrogen and give the blue color to medium denoted citrate utilization positive as reported by Panhwar *et al.*, 2012. This indicates that if the test is positive, the pH is raised and there will be no acid in the end product as reported by Park *et al.*, (2005).

The oxidase test is used to determine if a bacterium produces cytochrome c oxidase. The cytochrome system is usually present in aerobic organisms that are capable of using oxygen as the final hydrogen acceptor. The reagent is dark-blue to maroon in color when oxidized, and colorless when reduced. Maximum isolates of diazotrophic bacteria require aerobic conditions except few of them can grow even in anaerobic conditions as indicated by oxidase test. Similar results are also reported by Karpagam and Nagalakshmi (2014).

Table.1 Location of the soil samples

S. No.	Village	block	GPS Location
1.	Tinda	RS Pura	32°37'40.662"N 74°47'23.310"E
2.	Rattian	RS Pura	32°37'11.850"N 74°47'09.342"E
3.	Kir Pind	RS Pura	32°37'11.568"N 74°47'09.048"E
4.	Mahlawal	RS Pura	32°37'01.434"N 74°46'50.622"E
5.	Tanda	RS Pura	32°36'46.944"N 74°46'27.630"E
6.	Kotli Shah	RS Pura	32°36'56.796"N 74°46'06.198"E
7.	Mahlawal	RS Pura	32°37'03.300"N 74°45'52.020"E
8.	Tikrian	RS Pura	32°37'19.884"N 74°44'43.182"E
9.	Banota	RS Pura	32°37'19.734"N 74°44'41.988"E
10.	Langarwal	RS Pura	32°37'16.992"N 74°42'27.378"E
11.	Kulle	Bishnah	32°37'13.914"N 74°50'40.410"E
12.	Dhinda	Bishnah	32°37'06.498"N 74°50'48.810"E
13.	Bishnah Tehsil	Bishnah	32°36'58.716"N 74°50'57.354"E
14.	Ban Chak	Bishnah	32°36'24.256"N 74°52'09.522"E
15.	Pante Di Chubbian	Bishnah	32°36'05.634"N 74°53'13.776"E
16.	Sorer Tokor	Bishnah	32°35'47.604"N 74°53'34.026"E
17.	Chubbian Brahmana	Bishnah	32°35'44.736"N 74°52'59.346"E
18.	Chorli	Bishnah	32°35'56.058"N 74°52'27.822"E
19.	Dabbar	Bishnah	32°35'39.756"N 74°51'46.266"E
20.	Daali	Bishnah	32°35'52.932"N 74°50'07.632"E

Table.2 Enumeration of total biomass and N-fixers in Jammu district

Dilution(x10)	Total Biomass		Nitrogen fixers		% of N-fixer	
	R. S. Pura	Bishnah	R. S. Pura	Bishnah	R. S. Pura	Bishnah
1	110	108	33	30		
2	55	54	24	23		
3	42	40	20	19		
4	31	31	15	14	42.9	40.8
5	15	15	9	8		
6	8	7	6	6		
7	4	5	5	5		
8	2	1	2	2		
Grant total	266	262	114	107		

Table.3 Bacterial Morphology of Diazotrophic bacteria in rhizosphere soils in different areas of Jammu District

Medium	Bacteria	Size	Shape	Elevation	Surface	Consistency	pigmentation
<i>Becking</i>	<i>Azotobacter</i>	Small	Circular	Raised	Smooth	Viscous	Creamy white
<i>Becking</i>	<i>Beijerinckia</i>	Small	Irregular	Flat	Smooth	Viscous	White
<i>Nitrifying</i>	<i>N-fixers</i>	Pin head	Small	Circular	Round	Smooth	Off-White

Table.4 Characterization of diazotrophic bacteria (*Azotobacter*) from rhizosphere soil of Basmati-370 rice in different areas of Jammu District

Sr. No.	Sample	Gram staining	Cell morphology	Catalase test	Nitrate reduction test	Citrate utilization test	Oxidase test
1	R1	–	Rod shaped	+	+	+	+
2	R2	–	Rod shaped	+	+	+	+
3	R3	–	Rod shaped	+	+	+	+
4	R4	–	Rod shaped	+	+	+	+
5	R5	–	Rod shaped	+	–	–	+
6	R6	–	Rod shaped	–	+	+	+
7	R7	–	Rod shaped	+	–	–	+
8	R8	–	Rod shaped	+	+	+	+
9	R9	–	Rod shaped	+	–	+	–
10	R10	–	Rod shaped	+	+	+	+
11	B1	–	Rod shaped	+	+	+	+
12	B2	–	Rod shaped	–	+	+	–
13	B3	–	Rod shaped	+	+	+	+
14	B4	–	Rod shaped	+	–	+	–
15	B5	–	Rod shaped	+	+	–	+
16	B6	–	Rod shaped	–	+	+	+
17	B7	–	Rod shaped	–	–	+	+
18	B8	–	Rod shaped	+	+	–	+
19	B9	–	Rod shaped	–	+	+	+
20	B10	–	Rod shaped	+	+	+	+

R: I block, B: II block, 1-10: No of villages

Table.5 Characterization of diazotrophic bacteria (*Beijerincka*) from rhizosphere soil of Basmati-370 rice in different areas of Jammu District

Sr. No.	Sample	Gram staining	Cell morphology	Catalase test	Nitrate reduction test	Citrate utilization test	Oxidase test
1	R1	-	Rod shaped	+	+	+	+
2	R2	-	Rod shaped	+	+	-	+
3	R3	-	Rod shaped	+	-	+	+
4	R4	-	Rod shaped	+	+	+	+
5	R5	-	Rod shaped	+	+	+	+
6	R6	-	Rod shaped	+	+	+	+
7	R7	-	Rod shaped	+	-	-	+
8	R8	-	Rod shaped	+	+	+	-
9	R9	-	Rod shaped	+	+	-	+
10	R10	-	Rod shaped	-	+	+	+
11	B1	-	Rod shaped	+	+	+	+
12	B2	-	Rod shaped	-	+	+	+
13	B3	-	Rod shaped	+	-	+	+
14	B4	-	Rod shaped	+	+	+	+
15	B5	-	Rod shaped	+	+	+	+
16	B6	-	Rod shaped	-	+	-	-
17	B7	-	Rod shaped	+	+	+	+
18	B8	-	Rod shaped	+	-	+	-
19	B9	-	Rod shaped	+	+	+	-
20	B10	-	Rod shaped	-	+	+	+

Table.6 Isolation and characterization of diazotrophic bacteria (*Azotobacter*) from rhizosphere soil of Ratna rice in different areas of Jammu District

Sr. no.	Sample	Gram staining	Cell morphology	Catalase test	Nitrate reduction test	Citrate utilization test	Oxidase test
1	R1	–	Rod shaped	+	+	+	+
2	R2	–	Rod shaped	–	–	+	+
3	R3	–	Rod shaped	+	+	+	+
4	R4	–	Rod shaped	+	+	–	+
5	R5	–	Rod shaped	+	–	+	+
6	R6	–	Rod shaped	–	+	+	+
7	R7	–	Rod shaped	+	–	+	–
8	R8	–	Rod shaped	–	+	+	–
9	R9	–	Rod shaped	+	+	+	+
10	R10	–	Rod shaped	+	+	+	+
11	B1	–	Rod shaped	+	+	–	+
12	B2	–	Rod shaped	+	–	+	+
13	B3	–	Rod shaped	+	+	+	+
14	B4	–	Rod shaped	+	+	+	+
15	B5	–	Rod shaped	+	+	+	+
16	B6	–	Rod shaped	+	+	+	+
17	B7	–	Rod shaped	+	+	+	+
18	B8	–	Rod shaped	–	+	+	+
19	B9	–	Rod shaped	+	+	–	+
20	B10	–	Rod shaped	+	+	–	+

Table.7 Isolation and characterization of diazotrophic bacteria (*Beijerinckia*) from rhizosphere soil of Ratna rice in different areas of Jammu District

Sr. no.	Sample	Gram staining	Cell morphology	Catalase test	Nitrate reduction test	Citrate utilization test	Oxidase test
1	R1	-	Rod shaped	+	+	+	+
2	R2	-	Rod shaped	+	-	+	+
3	R3	-	Rod shaped	+	+	+	+
4	R4	-	Rod shaped	+	+	-	+
5	R5	-	Rod shaped	+	+	+	+
6	R6	-	Rod shaped	+	+	+	+
7	R7	-	Rod shaped	+	+	-	+
8	R8	-	Rod shaped	+	+	+	+
9	R9	-	Rod shaped	+	-	+	-
10	R10	-	Rod shaped	+	+	+	+
11	B1	-	Rod shaped	+	+	+	+
12	B2	-	Rod shaped when young, Dumbbell when mature	+	+	+	-
13	B3	-	Rod shaped when young, Dumbbell when mature	-	+	+	+
14	B4	-	Rod shaped	+	+	+	+
15	B5	-	Rod shaped when young, Dumbbell when mature	-	+	-	+
16	B6	-	Rod shaped	+	-	-	+
17	B7	-	Rod shaped when young, Dumbbell when mature	+	+	+	+
18	B8	-	Rod shaped	+	+	+	-
19	B9	-	Rod shaped	-	-	+	+
20	B10	-	Rod shaped	+	+	+	+

Table.8 Physico-chemical properties of the rhizosphere soils of rice in different areas of Jammu district

S. No	Parameters	Basmati-370	Ratna
1	Sampling depth	0-15	0-15
2	Moisture %	15.47±1.96*	14.40±2.61
3	Particle sizes	32.26	35.86
	Clay	27.78	27.64
	Silt	38.88	36.48
4	Textural class	Clay loam	Clay loam
5	pH	6.97±0.37*	6.90±0.34
6	EC	0.30±0.07*	0.28±0.04
7	OC	0.60±0.06*	0.58 ±0.04
8	Available N	159.6 ±27.91*	171.8 ±25.24
9	Available P	6.97 ±0.84*	7.05 ±0.72
10	Available k	75.6 ±17.19*	70.8 ±12.93

*±indicates S.D values

Physico-chemical properties

The pH of soils varied from slightly acidic to neutral. The pH of rhizosphere soil of Basmati-370 rice ranged from 6.28 to 7.50 having mean value of 6.97 whereas Ratna rice soils had a mean value of 6.90 in Jammu di strict (RS Pura and Bishnah blocks). The Electrical conductivity (EC) of rhizosphere soil ranged from 0.14 to 0.41 dS m⁻¹ in Basmati-370 and 0.22 to 0.38 dSm⁻¹ in Ratna rice respectively. These values are in safe range for rice cultivation. The organic carbon content lied in medium range. Organic carbon content of soils in Basmati-370 and Ratna ranged from 0.51 to 0.72 (%) and 0.51 to 0.65 (%), respectively. Highest organic carbon 0.72 % was recorded at Kotli Shah Village. The moisture content of rhizosphere soil lied between 11.94 to 19.66 % in Basmati-370 soils and 9.8 to 17.72 % in Ratna rice. The dominant soil textural class was clay loam. The clay content of rhizosphere soil ranged from 18.8 to 50.0 %, silt content ranged from 14.7 to 36.5 % and sand content ranged from 25.1 to 49.2 % in Basmati-370 rice. In rhizosphere soils of Ratna clay content ranged from 26.3 to 49.2 %, silt content ranged from 18.2 to 36.3 % and sand content varied from 14.5 to 46.6 %.

The available nitrogen content of soil in rice rhizosphere of Jammu district (RS Pura and Bishnah blocks) is in medium range. The available nitrogen in rhizosphere soil ranged from 129.2 to 212.2 mg kg⁻¹ with mean value 159.6 mg kg⁻¹ in Basmati-370, whereas it ranged from 129.4 to 221.7 mg kg⁻¹ with mean value 171.8 mg kg⁻¹ in Ratna rice of Jammu district. The available phosphorus content in rhizosphere soil ranged from 6.02 to 8.76 mg kg⁻¹ with the mean value of 6.97 mg kg⁻¹ in Basmati-370 and it ranged from 6.21 to 8.56 mg kg⁻¹ with the mean value 7.05 mg kg⁻¹ in Ratna rice. Phosphorus content in rice soils fell in medium range. The available potassium content in rhizosphere soil ranged from 60.5 to 105.1 mg kg⁻¹ with the mean value of 75.6 mg kg⁻¹ in Basmati-370, while it ranged from 60.5 to 93.5 mg kg⁻¹ with the mean value of 70.8 mg kg⁻¹ under Ratna rice of Jammu district (RS Pura

and Bishnah). The potassium content lies between low to medium range. The mean values of physico-chemical parameters are presented in (Table 8).

The presence of Nitrogen fixers in rhizosphere soils of basmati-370 rice variety highlighted increased nitrogen availability in the soil to fix atmospheric nitrogen. Changes in different environmental factors such as soil moisture, pH, EC, OC, N, P, K contents in different areas of R. S. Pura and Bishnah blocks have influenced nitrogen fixation in soil thus affecting nitrogen fixers in the soil. The study highlighted the aerobic and anaerobic nature of isolates based on biochemical tests viz. catalase, nitrate reduction, citrate utilization and oxidase tests performed on them.

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