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CHARACTERIZATION AND IDENTIFICATION OF PHYLLOSPHERIC BACTERIAL ISOLATES OF WHEAT (*Triticum aestivum*)

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ABSTRACT
Plant phyllosphere constitute a habitat for variety of microorganisms; bacteria are most common ones. Present investigation was carried out to isolate, characterize & identify bacterial isolates from the wheat phyllosphere at different locations of Ranchi districts. Reduction in wheat phyllospheric bacterial population was found at polluted site as compared to unpolluted site. Twenty four isolates were observed & examined on the basis of their morphological, physiological & biochemical characters for identification of bacterial isolates. Out of these, thirteen were found in unpolluted while, eleven were at polluted site. *Azotobacter* sp. was predominant in unpolluted site and their frequency decreased in polluted site. The genus *Pseudomonas* and *Beijerinckia* sp. were only found in unpolluted site. *Klebsiella* sp. showed resistance towards pollution and were dominant at polluted site.

KEY WORDS: Phyllosphere, wheat, Bacteria, Characters & Identification.

INTRODUCTION

Phyllospheric bacteria have agricultural and environmental importance; they can affect plant growth and can also support & stimulate the colonization, infection of tissues by plant pathogens (Rasche *et al*., 2006). It is frequently considered that the magnitude of bacterial population is an important indication of the fertility of the soil. Mwajita *et al*. (2013) evaluated the Rhizopsphere, Rhizoplane and Phyllospheric bacteria and fungi isolated from Rice in Kenya for PGPB and reported that over 50 % of bacterial isolates from phyllosphere were able to solubilize phosphates. Eventually the nitrogen fixing bacteria will supply the necessary combined nitrogen for the development and maintenance of the other phyllospheric population and the physiological state of the supporting foliage will be determining which of the micro-organisms will become dominant in the phyllosphere. The kinds of micro-organisms occurring in the phyllosphere population and the degree to which the leaf surface is covered vary for different plant species under different environmental condition. The occurrence of a microbial population rich in nitrogen-fixing organisms in phyllosphere of vegetation may be of decisive importance for the nutrition. Keeping these facts in mind, present investigation were done to isolate, characterize and identify wheat phyllospheric bacterial isolates on the basis of their morphological, physiological and bio-chemical properties.

MATERIALS & METHODS

Selection of location & sample collection

Leaf samples of wheat were collected from polluted (near road, industrial sites) and unpolluted (one km away) sites from six different locations of Ranchi district in Jharkhand.

Location selected for the leaf sample collection

Location	Area name	Geographical situations	Site name	
			Polluted site (PS)	Unpolluted site (UPS)
L1	Ranchi – Hazaribagh	23^0 22 N - $85^0$21 E	Road side (RSL1)	Remote side (RRL1)
L2	Ranchi – Jamshedpur	23^0 22 N - $85^0$21 E	Road side (RSL2)	Remote side (RRL2)
L3	Ranchi – Gumla	23^0 22 N - $85^0$19 E	Road side (RSL3)	Remote side (RRL3)
L4	HEC, Sector – Two	23^0 19 N - $85^0$17 E	Industrial site (ISL4)	Remote side (IRL4)
L5	HEC, Sector – Three	23^0 18 N - $85^0$17 E	Industrial site (ISL5)	Remote side (IRL5)
L6	Usha Martin, Ranchi	23^0 22 N - $85^0$25 E	Industrial site (ISL6)	Remote side (IRL6)

15 –20 leaves/plant from three plant replicates were taken randomly from the sites of all location. Matured whole leaf samples were removed with the help of sterilized scissors between 9.00 AM to 3.00 PM during bright sunshine and were kept separately in sterilized poly bags. The leaf samples were stored at low temperature (4 ^{0}C) till completion of the experiment.

Isolation and Identification of phyllospheric bacteria

Isolation

Counts of bacteria were done by serial dilution plate technique (Aneja, 2007) using nutrient agar medium. Discs of 1 cm diameter were excised from leaves with the help of a sterilized cork borer. Fifty such discs per sample were placed in a 250 ml conical flask containing 100 ml of sterilized distilled water. Flask was shaken vigorously for 15–20 minutes to detach the surface propagules. For

counting of bacteria 10^{-7} dilution were taken. Pure cultures of phyllospheric bacterial isolates were obtained through repeated streaking of well differentiable colonies on solidified media for microbial groups. Pure cultures thus obtained were maintained by frequent sub culturing on slants throughout experiment.

Characterization

During total bacterial count, the plate showing maximum number of colonies was taken for further investigation in all cases. Assignment of appropriate genera to the phyllospheric bacterial isolates were done based on morphological (Shape and arrangement of cells, size, gram stain, and colony size, colour & shape on agar), physiological (O_2 relation & motility) and biochemical (Ammonia production, Urease test, Catalase test, Starch hydrolysis, Casein hydrolysis, H_2S production, NO_3 reduction, Growth on N free media & Growth on PSB media) characters as described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

Morphological characters

For examination of size and shape, smears on slides fixed by gentle heat were stained with carbol fuchsin. Gram stained bacterial cells on clean micro slides were used for measurement of bacterial size (length and width) using ocular micrometer. 24 hours old cultures of bacteria were stained and examined under microscope for shape and arrangement of cells. Isolates were examined for Gram staining characters. Shape, size and colour of isolates colony under study were streaked and plate were incubated as needed and growth were checked after 18-24 hours (Chhonkar *et al.*, 2007) in which above observation were taken.

Physiological characters

To determine whether a culture is aerobic or not nutrient broth (containing 0.005% bromocresol purple) columns were prepared in culture tubes. Observations were recorded at 48 hr. interval for 7 days. Hanging drop technique was performed for demonstrating motility of bacteria using 12 hr. old nutrient broth culture (Aneja, 2007).

Biochemical characters

(i) Ammonia production- Culture was incubated in peptone broth for 5 days at small piece of filter paper with Nessler reagent was placed in upper part of culture tube. Test tube was warmed on a water bath at 50 - 60° C. Filter paper shown brown to black, was indicator of ammonia production.

(ii) Urease test - Urease test was performed on urea agar medium**.** Basal medium was poured in 90.0 ml quantities in flasks, autoclaved and cooled to 45^0C. 10 ml filter sterilized urea solution (20 %) was added to each flask, after mixing it was poured in 5 ml culture tubes. After solidification of medium in slanting position, tubes were inoculated with test bacterium, incubated (30 ± 2^0 C) and observations were recorded at regular intervals up to 15 days. Presence of urease gives red colour and for no urease yellow colour.

(iii) Catalase test - Catalase test was performed by adding H_2O_2 to trypticase soy agar slant. Culture medium was poured in culture tubes and sterilized by autoclaving. Test bacterium was inoculated in agar slant and inoculated at 35^0C for 24-48 hrs. observations were taken by adding 3 - 4 drops of H_2O_2 to flow over the growth of each slant culture and the reaction obtained was recorded. Presence of gas bubbles indicates catalase +ve test.

(iv) Hydrolysis of starch- It was performed by adding soluble starch (0.2 percent) in nutrient agar medium. pH was adjusted to 7.0 - 7.2 with the help of bromothymol blue indicator. The liquid was dispensed in test tube and sterilized. When cooled the liquid was incubated with culture and incubated at 30^0C. After incubation a small portion of liquid was treated with diluted iodine solution. No change in colour indicate complete hydrolysis, a brown colour indicates partial hydrolysis; a blue colour indicates that there was no hydrolysis of starch.

(v) Casein hydrolysis - Autoclaved and cooled basal medium was poured into sterilizes Petri plates, inoculated with culture. A single line streak inoculation across the surface of medium was produce and plates incubated for 2 -3 days at 37^0 C in an inverted position. A clear zone around streak showed a positive reaction while absence of clear area around the growth of an organism is a negative reaction.

(vi) Hydrogen Sulphate production test - Production of H_2S by different isolates was tested with the help of impregnated strips of filter paper with 10 % solution of lead acetate and dried. A lead acetate strip was placed at the top of a nutrient broth culture and incubated. Blackening of paper indicated H_2S production.

(vii) Nitrate reduction - Nitrate broth was used with following composition: Peptone - 10.0 g, Beef -extract- 5.0g, KNO_3 - 1.0g, Distilled water-1000.0 ml. The ingredients were dissolved and poured in tubes (5 cm) and autoclaved. The broth was inoculated with the test bacterium and incubated at 25^0C. Reduction of nitate was checked upto 15 days at regular intervals by adding a few drops of sulfanilic acid (0.8 % in 5 M acetic acid) and dimethyl alpha-nephthylamine (0.5 % in 5 M acetic acid) to the nitrate broth culture subsequently. Presence of nitrite was detected by the red Colour given by addition of sulfanilic acid, –naphthylamine reagent to a portion of the liquid.

Determination of specific properties

Nitrogen fixing bacteria - For the assessment of Nitrogen -fixing bacteria, Burk's Nitrogen free medium was used and incubated in 250 ml conical flasks inoculated with culture of test organisms. The flasks were then incubated for 14 days at 30^0C. After the incubation, the contents of the flasks along with uninoculated contents as control were digested for the determination of nitrogen by the Kjeldahl's method (Jackson, 1973).

Phosphate solubilizing bacteria - For the assessment of P – solubilizing bacteria, Bunt and Rovira (1955) medium was used. 100 ml of the medium replacing Tricalcium Phosphate by Rock Phosphates equivalent to 50 mg % P_2O_5 in 250 ml conical flasks were inoculated with different isolates. Inoculated flasks were incubated for 15 days at $30 \pm 0.2\ ^0$C under static condition and shaken once in 12 hr. along with control un-inoculated flasks. 5 ml of growth medium from each flask was withdrawn aseptically after 15 days and passed through Whatman No. 42 filter paper. The filtrates were assayed for P_2O_5 by Amino-Molybdate Ascorbic acid blue method (Jackson, 1973).

By comparing the results of the above tests as described in Bergey's Manual of Determinative Bacteriology (Holt, *et*

al., 1994), isolates was tentatively identified up to generic level.

RESULTS & DISCUSSION

Bacterial population

Bacterial population of wheat phyllosphere at different locations is presented in (Table 1). It is evident from the data that population of bacteria decreased significantly in polluted site as compared to unpolluted sites of wheat phyllosphere. Population of wheat phyllosphere was reduced to extent of 41.57%. The reduction in bacterial colonization located in the vicinity of industries and roadsides may be attributed to increased absorption of heavy metals, dust, traffic fumes. Location L3 & L5 recorded significantly higher bacterial count. Our results are agreement with the findings of Marta *et al.* (2009).

Characteristics of bacterial isolates

The bacterial isolates found on wheat phyllosphere from different locations have been placed in seven different groups on the basis of morphological, physiological and biochemical characteristics (Table, 2a and 2b).

Group (A) Cells of this group of isolates were short rods, rods, occurring in pairs and chains, individual cells ranged from 0.7μ to 2.0μ in length and 2.5μ to 4.5μ in width, size of colony on nutrient agar medium varied between 6 to 15 mm diameter, yellow, brown and white in colour, shape of these isolates scattered, irregular, raised, granular and wrinkled. Physiologically they are Gram negative, positive growth with pressure of oxygen and motile. Biochemically these isolates produced ammonia, hydrolyzed urea and reduced NO_3^-, but did not produced H_2S. Cells showed positive growth on inorganic nitrogen free medium. On the basis of above characteristics, isolates ($RRL1_1$, $RSL3_1$, $IRL4_2$, $IRL5_1$ and $IRL6_2$) assembled in one group and tentatively identified as members of genus *Azotobacter* sp.

Group (B) The cells were straight rod with round ends, pairs with diameter ranging 3.5 μ to 4.0 μ, colony size on agar 7 to 10 mm diameter, colour of these isolates varied white to shiny white in shape raised to highly raised, glistening, smooth. These isolates aerobic in nature of growth and Gram negative with motile. Urea hydrolysis and catalase test was found positive, but starch hydrolysis and nitrate reduction were negative. Cells showed inorganic phosphate solubilization on medium. Isolates $RRL1_2$, $IRL4_1$ and $IRL5_2$ resembling in above characters were placed and tentatively identified as members of genus *Pseudomonas* sp.

Group (C) Cells bearing straight rods, short rods occurring in there singly in pairs or chains. Individual cells ranged from 0.6 μ to 1.5 μ in length and 1.2 μ to 2.5 μ in width, colony on agar medium varied in size from 8 to 12 mm, light yellow to yellow, shape coarsely granular, raised, smooth. Physiologically they showed aerobic nature of growth with Gram positive motile. Ammonia production, urease, catalase and nitrate reduction positive, while starch and H_2S production negative. On the basis of above characteristics, organisms $RSL1_1$, $RSL2_1$ and $ISL4_1$ may tentatively be identified as to genus *Aminobacter* sp.

Group (D) Spherical shaped cells with diameter ranging between 4.0 μ to 5.0 μ. size of colony on nutrient agar 5 to 15 mm, white to yellowish in colour, circular, flat, raised, smooth. They were aerobic, Gram positive and non-motile. Urea hydrolysis, catalase test and H_2S production was found positive, but starch hydrolysis and NO_3^- negative. Cells showed positive growth on inorganic nitrogen free medium. These organisms $RSL1_2$, $RRL3_1$, $RSL3_2$ and $ISL5_1$ with above characters were tentatively identified as the genus *Klebsiella* sp.

TABLE 1. Bacteria population cm^{-2} (x 10^7) on *Triticum aestivum* phyllosphere at different location

Locations/	*Triticum aestivum*		
Crops	UPS	PS	Mean
L1	16.0	8.8	12.4
L2	20.9	9.2	15.1
L3	18.6	14.0	16.3
L4	19.0	10.0	14.5
L5	18.9	13.7	16.3
L6	20.7	11.1	15.9
Mean	19.0	11.1	15.1
	L	P	L x P
S.Em. ±	0.53	0.18	1.26
C.D. 5%	1.10	0.37	NS
C.V. %		5.04	

UPS – Un-polluted site, PS – Polluted site.

Group (E) Cells having ellipsoidal shape, diameter ranging between 0.5μ to 1.0μ in length and 1.2 to 3.0 in width, size of colony 5 to 10 mm, white to dull white in colour, shape varied circular, smooth, slimy and irregular. Physiological natures of the isolates were aerobic, Gram negative and motile. Biochemically they were produced ammonia and H_2S, similarly, hydrolyzed urea and starch, but did not reduced NO_3^-. Positive growth of cells on mineral nitrogen free medium. On the basis of above parameters, isolates ($RRL2_1$, $IRL3_3$ and $IRL6_1$) tentatively identified as belonging to genus *Beijerinckia* sp. Group (F) Rod shaped cells in chains with square ends. Individual cells ranged from 1.0 μ to 2.5 μ in length and 3.0μ to 4.5μ in width, 5 to 12 mm colony size on agar medium, creamy white to yellow, convex and entire edges growth, aerobic nature showed with pressure of oxygen and Gram positive with non - motile. Production of ammonia, hydrolysis of starch, casein liquefied and catalase was positive.

TABLE 2(a): Morphological & Physiological characteristics of bacterial isolates & their identification of phyllospheric bacterial isolates

Location	Isolate no.	Morphological					Physiological		
		Shape and arrangement of cells	Size, µm	Colony on agar			Gram stain	O_2 relation	Motility
				Size, mm	Colour	Shape			
RRL1	1^{A}	Rods, pairs	0.7 x 2.5	12	White	Wrinkled, raised	–	Aerobic	+
	2^{B}	Straight rods, round ends	4.0	10	White	Raised, glistening	–	,,	+
RSL1	1^{C}	Rods, pairs	0.6 x 2.5	12	Yellow	Smooth, raised	+	,,	+
	2^{D}	Spherical	5.0	10	White	Circular, flat	+	,,	–
RRL2	1^{E}	Ellipsoidal	0.5 x 1.7	5	Dull white	Irregular, smooth	–	,,	+
	2^{F}	Rods, square ends in chain	1.0 x 2.0	10	Creamy White	Convex, entire edges	+	,,	–
RSL2	1^{C}	Straight rod, single chain	1.0 x 1.2	8	Light yellow	Circular, smooth, raised	+	,,	+
	2^{F}	Rods, square ends	0.5 x 1.5	7	Yellow	Round, entire edges	+	,,	+
RRL3	1^{D}	Spherical	4.5	7	Yellowish	Circular, raised	+	,,	–
	2^{F}	Short rods, square ends	0.6 x 1.0	12	Yellow	Entire edge, translucent, opaque	+	,,	–
	3^{E}	Ellipsoidal	0.7 x 1.2	10	White	Circular, smooth, slimy	–	,,	+
RSL3	1^{A}	Rods, chain	2.0 x 3.0	12	Brown	Irregular, raised	–	,,	+
	2^{D}	Spherical	4.5	15	White	Circular, flat, smooth	+	,,	+
IRL4	1^{B}	Straight, pairs	3.5	8	White	Highly raised, glistening, smooth	–	,,	+
	2^{A}	Rod, singly in chains	1.0 x 3.0	8	Yellow	Raised, Irregular, granular	–	,,	+
ISL4	1^{C}	Straight rod, single chain	1.5 x 2.0	12	Yellow	Coarsely granular, circular, raised	+	,,	–
	2^{G}	Cocci	0.8	12	Yellow	Coarsely granular, raised, smooth	–	Anaerobic	–
IRL5	1^{A}	Straight rods, chains	1.2 x 4.5	6	Yellow	Raised, Irregular	–	Aerobic	+
	2^{B}	Straight rods, pairs	4.0	7	Shiny white	Raised, glistening, smooth	–	,,	+
ISL5	1^{D}	Spherical	4.0	5	Yellowish	Circular, flat	+	,,	+
	2^{F}	Rods, square ends	1.2 x 2.0	5	Yellow	Round, convex, entire edges	+	,,	–
IRL6	1^{E}	Ellipsoidal,	1.0 x 3.0	5	Dull white	Circular, slimy	–	,,	+
	2^{A}	Rod, singly in pairs	1.5 x 3.5	15	Yellow	Raised, scattered	–	,,	+
ISL6	1^{G}	Cocci	4.4	10	Deep yellow	Granular, raised	–	Anaerobic	–

+ Positive, – Negative.

TABLE 2(b): Biochemical characteristics and identification of wheat phyllospheric bacterial isolates

Location	Isolate no.	Ammonia production	Urease test	Catalase test	Starch hydrolysis	Casein hydrolysis	H_2S production	NO_3 Reduction	Growth on N free media	Growth on PSB media	Identified Genus
RRL1	1^{A}	+	+	–	–	–	–	+	+	–	*Azotobacter*
	2^{B}	–	+	+	+	–	–	–	–	+	*Pseudomonas*
RSL1	1^{C}	+	+	+	–	–	–	+	–	–	*Aminobacter*
	2^{D}	–	+	+	–	–	+	–	+	–	*Klebsiella*
RRL2	1^{E}	+	+	–	+	–	+	–	+	–	*Beijerinckia*
	2^{F}	+	–	+	+	+	–	–	–	+	*Bacillus*
RSL2	1^{C}	+	+	+	–	–	–	+	–	–	*Aminobacter*
	2^{F}	+	–	+	+	+	–	–	–	+	*Bacillus*
RRL3	1^{D}	–	+	+	–	–	+	–	+	–	*Klebsiella*
	2^{F}	+	–	+	+	+	–	–	–	+	*Bacillus*
	3^{E}	+	+	–	+	–	+	–	+	–	*Beijerinckia*
RSL3	1^{A}	+	+	–	–	–	–	+	+	–	*Azotobacter*
	2^{D}	–	+	+	–	–	+	–	+	–	*Klebsiella*
IRL4	1^{B}	–	+	+	+	–	–	–	–	+	*Pseudomonas*
	2^{A}	+	+	–	–	–	–	+	+	–	*Azotobacter*
ISL4	1^{C}	+	+	+	–	–	–	+	–	–	*Aminobacter*
	2^{G}	–	+	+	–	–	–	+	–	–	*Phyllobacterium*
IRL5	1^{A}	+	+	–	–	–	–	+	+	–	*Azotobacter*
	2^{B}	–	+	+	+	–	–	–	–	+	*Pseudomonas*
ISL5	1^{D}	–	+	+	–	–	+	–	–	–	*Klebsiella*
	2^{F}	+	–	+	+	+	–	–	–	+	*Bacillus*
IRL6	1^{E}	+	+	–	+	–	+	–	+	–	*Beilerinckia*
	2^{A}	+	+	–	–	–	–	+	+	–	*Azotobacter*
ISL6	1^{G}	–	+	+	–	–	–	+	–	–	*Phyllobacterium*

+ Positive, – Negative.

However, the isolates failed to produce H_2S and reduced nitrate. Cells solubilized inorganic phosphate on medium. Isolates $RRL2_2$, $RSL2_2$, $RRL3_3$ and $ISL5_2$ resembling in above characters with placed in one group and tentatively being identified as members of genus *Bacillus* sp.

Group (G) Cells of this group isolate were coccoid with diameter ranging between 0.8µ to 4.4µ, Gram negative, colony size on agar 10 to 12 mm, yellow to deep yellow in colour, coarsely granular to granular, circular and raised. They were anaerobic, Gram negative and non-motile. Biochemical nature of isolates showed urea hydrolysis, catalase test and nitrate reduction was positive. However, H_2S production and starch hydrolysis was found negative. On the basis of above parameters, organisms ($ISL4_2$ and $ISL6_2$) tentatively identified as the genus *Phyllobacterium* sp. Twenty four isolates were observed and examined for cultural and biochemical characterization. Unpolluted site recorded thirteen isolates, however, eleven isolates were observed at polluted site. Less number of isolates observed at polluted site as compared to unpolluted site might be due to sensitivity of bacterial isolates towards the pollution (Brighigna *et al.,* 2000). *Azotobacter* sp. was predominant in unpolluted site. *Klebsiella* sp. showed resistance towards pollution and were dominant at polluted site. Genus *Pseudomonas* and *Beijerinckia* sp. were only reported in unpolluted site. Present investigations are in agreement with Brighigna, *et al.* (1999).

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