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MICROBIAL DIVERSITY OF RHIZOSPHERIC SOIL WITH SPECIAL REFERENCE TO PLANT GROWTH PROMOTING ISOLATES OF AZOTOBACTER

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ABSTRACT

Soil microbial populations are the key element in recycling the plant nutrients and thus play a major role in the maintenance of soil fertility and soil health. Various rhizospheric and non-rhizospheric soil samples of agricultural fields of Aligarh revealed the significant viable plate count of various functional groups of soil microorganisms. The population density of aerobic heterotrophic bacteria, actinomycetes, filamentous fungi did not significantly differ in the different field soil irrespective of crop under cultivation. Rhizospheric soil yielded almost ten times more microbial biomass as compared to non-rhizospheric soils. On the other hand population density of aerobic asymbiotic nitrogen fixers particularly *Azotobacter chroococcum* was poor in soil receiving long-term application of wastewaters. A total of 48 isolates of *Azotobacter* recovered from different crop rhizospheric soils were tentatively grouped into three morphological types on the basis of pigmentation and cultural characteristics. Isolates of group -A was tentatively identified as *A. chroococcum* and group B & C as the other species of *Azotobacter.* Carbohydrates like glucose, fructose, lactose, maltose, adonitol and rhamnose were commonly utilized by the fair number of isolates. Antibiotic resistance profile against 8 antibiotics revealed 60.4% strains resistant to nitrofurantoin followed by nalidixic acid (58.3%), co-trimazine (37.5%), novabiocin (31.2%), cloxacillin (20.8%) and least to chloramphenicol (12.5%). All test strains were found sensitive to streptomycin and doxycycline. All isolates produced ammonia, and showed varying degree of positive influence on germination of moong seeds *in vitro*. HCN production could be detected in five isolates. IAA production in the selected isolates varied from 3.0 to 7.6 µg/ml of culture filtrate. These isolates showed tolerance to salt (2.5-3.5% NaCl conc.) and pH value (7-10). It is expected that isolates exhibiting multi-PGP activities and elevated tolerance to environmental factors may be suited for further assessment and development as the effective PGPR- inoculants.

Keywords*:* Azotobacter, antibiotic resistance, plant growth promoting activity, Indole acetic acid and tolerance traits.

INTRODUCTION

The rhizospheric soil is densely populated with microorganisms because it is rich in microbial nutrient as a result of root exudation. The microbial populations interact with plants as well as with themselves. The Rhizospheric bacteria may release various plant growth promoting substances as a secondary metabolites, which may promote the plant growth directly or indirectly. Such beneficial rhizospheric bacteria are often referred as plant growth promoting rhizobacteria. (PGPR). *Pseudomonas* is one of the extensively studied PGPR, which promote the growth of plants by protecting the plant health from soil borne phytopathogens and their toxins. The PGP activity associated with this bacterium are production of antibiotic, siderophores, HCN etc. On the other hand several other bacteria may promote plant growth directly by producing plant growth harmones and other substances in close contact to plant root. The list of such bacteria is long however other potential bacteria include asymbiotic nitrogen fixers, phosphate solubilizer's in addition to well studied organism like fluorescent *Pseudomonas* and certain species of *Bacillus*[1].

It has been assumed that inoculation with nitrogen fixing bacteria like *Rhizobium, Azotobacter* and *Azospirillum* enhanced plant growth as result of their ability to fix nitrogen. However, despite extensive research efforts, only rhizobia have been shown to increase the yields from dinitrogen fixation. In many cases, nitrogen levels in plant tissues and soil do not increase upon inoculation with these bacteria. Growth

promotion may therefore be attributed to other mechanism such as production of plant growth regulating substances (PGRs). Thus an inoculum should be screened for the production of PGRs in addition to its primary activity[2]. Environmental factors like pH, salinity, and physicochemical properties of soil may also influence the survival, colonization, activity and field performance of an inoculum. *Azotobacter chroococcum* is a well known free living nitrogen fixing bacterium and capable of synthesizing and secrete plant growth promoting substances like thiamin, riboflavin, IAA, gibberellin etc., and frequently used as nitrogenous biofertilizer for a number of crops[3]. However, the incidence and diversity of free living asymbiotic nitrogen fixing bacteria including *Azotobacter chroococcum* in terms of their PGP properties and tolerance traits to environmental factors are poorly studied[4-5]. It is expected that mapping of particular area for indigenous *Azotobacter* and related bacteria for their diversity and above desirable characteristics may yield more effective and adaptive strains to be developed as bio-inoculant. Thus isolation and screening of indigenous *A. chroocoocum* and related bacteria for multiple PGP activities and tolerance to environmental factors may provide more effective inoculant strains. Such strains if needed may be further improved in their performance by routine genetic manipulation (mutation and gene transfer methods). In the present investigation an attempt has been made to explore the diversity of free living nitrogen fixers population in the rhizospheric soils of different crops and examine commonly occurring Azotobacter and related species for their PGP activities and tolerance to antibiotic, salt and pH.

MATERIAL AND METHODS

Enumeration of soil microbial diversity

Composite soil samples of rhizospheric soil of various crops and different location in the vicinity of Aligarh from mainly agricultural field were collected in sterile polythene bags gram of the thoroughly mixed suspension of soil in sterile distilled water was further serially diluted in normal saline solution and spread on the nitrogen free Jensen's agar plate (Sucrose 20g, dipotassium hydrogen phosphate 1g, magnesium sulfate 0.5g, sodium molybedate-0.005g, sodium chloride 0.5g, ferrous sulfate 0.1g, calcium carbonate 2g, agar 20g/litre) in duplicate. The inoculated plates were incubated for 72 hrs. or more at 28°C. Plates were checked for *A. chroococcum* growth and specific pigmentation on prolong incubation. Total viable count of the asymbiotic nitrogen fixers and *A. chroococcum* were determined in term of c.f.u. / gm of soil by spread plate method. The well-isolated colonies of *A. chroocoocum* were purified and maintained on slants. The isolates were identified according to specific characteristics described in the Bergey's manual of determinative bacteriology (1985)[6] and stored at 4°C on slants. Three predominant colonial forms of the isolated bacteria were grouped as A, B and C category of diazotrophs associated with rhizospheric soils. Similarly plate viable count of soil aerobic hetrotrophic bacteria, actinomycetes, asymbiotic nitrogen fixers and filamentous fungi were determined using standard plate count method on their respective nutrient media as described elsewhere[5].

Biochemical characterization

The isolates were tested for their cultural and biochemical characteristics which include colony morphology, pigmentation, gram-staining reaction, production of polysaccarides, utilization of carbohydrates (sucrose, glucose, fructose and lactose) and citrate, production of catalase, indole and urease and reduction of nitrate by standard methods described by Cuppucino and Sherman[7]

Antibiotic sensitivity of test strains

Antibiotic sensitivity of the test isolates was carried out against eight antibacterial drugs by disc diffusion method described by Baur *et al.* as adopted earlier (Ahmad *et al.*).The antibiotic discs were obtained from Hi-media laboratory, Mumbai. The potency of discs was as follows. Cloxacillin, co-trimazine, doxycycline, nitrofunrantoin and nalidixic acid (30 mg/disc each), novobiocin, streptomycin and chloramphenicol (25mg/disc each). *E. coli* B (an antibiotic sensitive strain) was used as antibiotic disc potency control.

Screening of isolates for their plant growth promoting (PGP) activities

All the test strains of diazotrophic bacteria were assayed for their PGP activities like production of NH_3, Indole acetic acid according to the method described by Gordon and Weber[10]. Production of HCN was detected using standard method as described by Lork[11] and seed germination test by method of Shende *et al*.

Determination of salt and pH tolerance

Tolerance of the isolated diazotrophic bacteria against salt was determined by agar dilution method. Sodium chloride ranging from 0.5% to 3.5% was incorporated in the sterilized Ashby's agar medium at 45°C and poured into

plates. Each plate was divided into eight equal sectors and spot inoculated in duplicates, using freshly grown and appropriately diluted (10^5 CFU/ml) cultures of test strains with 5 mm inoculating loop. Plates were incubated at 28±2°C for 72 hrs or more. The concentration of salt that permitted mottled growth (few colonies developed) and beyond which there was no growth, was considered as minimum inhibitory concentration (MIC) of salt against test strain. Similarly, to determine the pH tolerance the pH of Ashby's medium was adjusted with the help of 1N NaOH from 7 to10. The spot inoculation method, described above was opted for determining the pH tolerance in term of MIC among test strain.

RESULTS AND DISCUSSION

Soil microbial diversity may be studied by different approaches. One approach is to study the total viable population of major groups of microorganisms to get a general picture about microbial diversity and soil health. In the present investigation, major microbiological populations like aerobic heterotrophic bacteria, actinomycetes, asymbiotic N_2-fixers (including *Azotobacter sp*.) and filamentous fungi were enumerated by plate count method on their respective media. Similarly specific soil bacterium *Azotobacter* was also enumerated in different rhizospheric soils as well. The findings clearly indicated that the distribution and occurrence of these major groups of microbial populations did not differ significantly among various sources of soil samples of Aligarh. However, as expected, viable count in soils was observed maximum for aerobic heterotrophic bacteria followed by actinomycetes, fungi and asymbiotic N_2-fixers (Tables1& 2). Varying frequency of occurrence of these microbial populations in rhizospheric and non-rhizospheric soils were also described by other workers[5,13-14]. The variation is probably due to the different soil ecological and

Table -1: Microbiological diversity of Aligarh soil

Sources of soil samples	Microbial diversity (CFU/gm of soil)*		
	Aerobic heterotrophic bacteria	Actinomycetes	Fungi
Pea field	3.5×10^7	1.2×10^6	3.2×10^5
Wheat field	5.4×10^7	2.9×10^5	5.4×10^5
Sugarcane field	7.5×10^7	1.8×10^6	3.7×10^5
Garden soil	1.9×10^7	1.0×10^6	1.6×10^5
Mustard field	2.5×10^6	3.5×10^5	1.2×10^5

* Values are of the mean of 3 – 5 samples
CFU = colony forming units

Table - 2 : Occurrence of free living asymbiotic diazotrophic bacteria in the rhizospheric and non-rhizospheric soils

Sources of soil sample	Frequency of occurrence			
	Asymbiotic nitrogen fixers ($\times 10^4$)		*Azotobacter chroococcum* ($\times 10^3$)	
	Non-rhizosphere	Rhizosphere	Non-rhizosphere	Rhizosphere
Pea	7.2	61	6.6	70
Cabbage	5.7	56	4.7	45
Sugarcane	6.8	63	7.3	49
Tomato	7.0	74	6.7	65
Wheat	5.6	42	5.8	42
Mustard (Sewage+ industry wastewater)	0.15	1.2	0.25	2.4
Mustard(Thermal power wastewater	1.1	9.4	2.2	16.5
Garden soil	2.3	ND	0.82	ND

Values are of the mean of 5 composite sample Jan- March 2002; ND= Not done

environmental conditions of the test samples. *Azotobacter* population was found in the range of $5.6x10^2$ to $7.3x10^3$ CFU/gm of soil. It is also noteworthy that *A. chroococcum* was least detected in the soil receiving long- term application of sewage and industrial effluents. However, other groups of asymbiotic N_2-fixers were detected. In general viable count of the rhizospheric soil is higher as compared to non- rhizospheric soil. This difference is expected due to the rich nutrient availabity in the rhizospheric zone contributed by plant root exudates[13].

All strains were found to be gram-ve, bacillary to oval shaped cells. Biochemical characteristics of three groups of diazotrophs showed certain common characteristics but varied in other as depicted in Table - 3. Isolates of group-A were positive for production of catalase (100%), urease (25%) and indole (30%) while 65% and 80% strains were positive for nitrate reduction and citrate utilization tests respectively. Among group-B isolates, maximum number of strains was positive for catalase test (93%) followed by citrate utilization (79%), nitrate reduction (64%) and indole production (50%), and least were positive for urease test (21%). Among Group-C isolates, the percent of positive strains were 86%, 79%, 64%, 43% and 36% for citrate, catalase, nitrate, indole and urease tests respectively (Table-3). All isolates of group-A utilized, glucose and maltose by whereas fructose, adonitol, rhamnose and lactose were utilized by 95%, 90%, 85% and 55% of the strains respectively. Similarly, among Group-B isolates, all strains utilized adonitol, 93% strains showed growth on maltose and rhamnose; whereas glucose, lactose and fructose supported growth for 86%, 79% and 72% of the strains respectively. The respective percentage of Group-C isolates for the utilization of adonitiol, rhamnose, maltose and lactose were found to be 93%, 86%, 79% and 79%, where as glucose and fructose supported growth for 64% and 57% strains respectively (Table-3). Thus further differentiation of strains of the three groups was achieved, but not sufficiently enough for all strains. However, these biochemical reports were similar

Table - 3: Biochemical characteristics of asymbiotic diazotrophic bacteria isolated from rhizospheric soils

Major characteristics	**Group-A**	**Group-B**	**Group-C**
Normal growth on nitrogen free medium	+	+	+
Gram reaction	-ve	-ve	-ve
Cell shape	Ovoid	Rod to ovoid	Rod - coccoid
Colony morphology	Large with irregular margin	Whitish, bluish white large round colonies	Large,watery, semitranslucent or transparent muciligenous colonoies
Pigmentation	Black- brown	Yellow-green, few fluorescent	No pigmentation
Polysaccarides production	+	+	+
Utilization of carbohydrate (% +ve isolates)			
Glucose	100	86	64
Fructose	95	72	57
Maltose	100	93	79
Lactose	55	79	79
Adonitol	90	100	93
Rhamnose	85	93	86
Biochemical test (% +ve strains)			
Indole production	30	50	43
Citrate utilization	80	79	86
Nitrate reduction	65	64	64
Catalase test	100	93	79
Urease test	25	21	36

Table- 4: Incidence of antibiotic resistance in the isolates free living diazotrophic bacteria

Antibiotics	Number of resistant strains			Total No. of resistant strains (%)
	Group - A20*	Group - B14*	Group - C14*	
Nitrofurantoin (Nf)	15	3	11	29 (60.4)
Nalidixic acid (Na)	4	12	12	28 (58.3)
Co-trimazine (Cm)	8	4	6	18 (37.5)
Novabiocin (Nv)	10	3	2	15 (31.2)
Cloxacillin (Cx)	1	4	5	10 (20.8)
Chloramphenicol (C)	Nil	2	4	6 (12.5)
Streptomycin (S)	Nil	Nil	Nil	Nil
Doxycycline (Do)	Nil	Nil	Nil	Nil

*Total number of isolates

with the results published in Bergeys Manual of Determinative Bacteriology[6] and general characters of *Azotobacter as* described by Subba Rao[3]. On the basis of specific biochemical tests, carbohydrate utilization, pigmentation, etc., group A organisms were tentatively identified as *A. chroococcum* while groups-B & C also belonged to the genus *Azotobacter*. Species level identification of these groups (B&C) requires additional biochemical and genetic characterization. Therefore, they were tentatively named as *Azotobacter* sp. I and *Azotobacter* sp.II respectively. These three groups of asymbiotic diazotrophs were predominantly occurring in Aligarh soils. Forty eight isolates of these groups (group-A 20 , group B 14 and group-C 14 isolates) were recovered from rhizospheric soil samples of 7 different crop plants viz. tomato, cabbage, sugarcane, wheat, mustard and pea (data not shown).

Antibiotic sensitivity behaviour against eight antibiotics revealed 60.4% resistance against nitrofurantoin followed by nalidixic acid (58.3%), co-trimazine (37.5%), novabiocin (31.2%), cloxacillin (20.8%) and least to chloramphenicol (12.5%). All test strains were found sensitive to streptomycin and doxycycline. Variation in the sensitivity patterns of the three groups was helpful in strain differentiation to some extent (Table-4). Varying levels of antibiotic resistance among diazotrophic bacteria was reported other workers[9,15]. Antibiotic resistance profile indicated that most of the isolates probably developed resistance by mutation as the plasmid encoded resistance to nalidixic acid, nitrofurantoin has not yet described. While in some isolates involvement of plasmid in mediating resistance to co-trimazine, chloramphenicol and cloxacillin could not be ruled out.

The test isolates were further screened for their plant growth promoting (PGP) activities like production of ammonia, HCN, indole acetic acid and promoting seed germination. The isolated strains differed in their PGP activity and antibiotic resistance patterns (Table - 5). It was interesting to note that all the strains produced ammonia. Earlier reports indicated ammonia production by 15-20% of *Azotobacter* population[16-17]. High level of HCN production was observed in five isolates (AZ-16, NAZ-13 and NAZW-5, 6, 12). Production of HCN and antifungal substances by PGPRs might improve the growth of plants indirectly through the suppression of soil borne pathogens[1].

Another plant growth promoting activity of these strains was evaluated by seed germination test *In vitro* effect of the inoculation of these strains on the germination of seeds of *Vigna radiata* (Var.T-44) revealed that the strains No. AZ-8, AZ-9, AZ-15 & AZ-18 of Group-A influence seed germination (>80%) than control (60%). The promotion of seed germination and emergence perhaps involves the action of emergence promoting rhizobacteria-EPR[18].

Further ten strains, which showed significant production of ammonia, HCN, and high seed germination percentage, were selected to test indole acetic acid (IAA) production. IAA production ranged from 3.0 to 7.6 µg/ml of culture filtrate. However, increased level of production was detected in two strains, AZ-8 & AZ-9. Our findings clearly indicated that IAA production was a common characteristic of *A. chrooroccum* and level of production was found to be strain dependent. Varying levels of IAA production by *Azotobacter* isolates have been reported in literature[19].

Table - 5: Antibiotic resistance pattern and some PGP activities of the test isolates

Strain code	Antibotic resistance pattern	Plant growth promoting activities			
		Ammonia production	IAA production (µg/ml)	HCN production	%Seed germination
AZ-1	Cm, Nv, Nf, Na	++	NT	-	++
AZ-2	Cm, Nv, Nf, Na	++	NT	+	++
AZ-3	Nf	+++	7-8	++	+++
AZ-4	Cm, Nv, Nf	++	NT	-	++
AZ-5	Nf	+++	7.2	+	+++
AZ-6	Nf	+++	4.2	++	+++
AZ-7	Nv, Nf	++	NT	-	+++
AZ-8	Nv, Nf	+++	7.3	+	+++
AZ-9	Nv, Nf	+++	7.6	++	+++
AZ-10	Nv	+	NT	-	++
AZ-11	Nv, Nf	+++	5.0	++	+++
AZ-12	Nf	+	NT	+	+++
AZ-13	Nf	++	NT	+	++
AZ-14	Cm, Nf	+	NT	+	++
AZ-15	Cm, Nv, Nf, Na	+	5.2	-	+++
AZ-16	Cm, Nf	++	6.2	+++	+++
AZ-17	Cx	++	NT	++	++
AZ-18	Sensitive	+	3.1	+	+++
AZ-19	Cm, Nv, Nf	+	NT	-	+++
AZ-20	Cm, Nv	+	4.3	+	++
NAZ-1	Cx, Na	+	NT	-	++
NAZ-2	C, Cx, Nf	++	NT	++	++
NAZ-3	Na	++	5.7	++	+++
NAZ-4	Na	++	3.8	-	+++
NAZ-5	Cm, Nv, Na	+	6.0	-	+++
NAZ-6	Cm, Nv, Na	+++	NT	+	+
NAZ-7	Sensitive	+++	NT	+	++
NAZ-8	C, Cm, Cx, Nv, Nf	++	NT	-	+
NAZ-9	Na	++	4.4	-	++
NAZ-10	Cm, Nf	++	NT	++	+++
NAZ-11	Na	+	NT	+	++
NAZ-12	Cx, Na	++	NT	+	+
NAZ-13	Na	++	NT	+++	++
NAZ-14	Na	++	NT	+	++
NAZW-1	Cx, Nf, Na	+	NT	-	++
NAZW-2	Cx, Nf, Na	+	NT	-	++
NAZW-3	Na	+	NT	-	++
NAZW-4	C, Cm, Cx, Nf, Na	++	NT	+	+++
NAZW-5	Nv, Na	++	1.2	+++	+++
NAZW-6	Cm, Cx, Na	++	5.6	+++	+++
NAZW-7	C, Cm, Nv, Nf, Na	+	1.4	+	++
NAZW-8	Cm, Nf, Na	++	NT	+	++
NAZW-9	Nf	++	NT	++	++
NAZW-10	Nf	++	NT	+	+++
NAZW-11	Nf, Na	++	6.9	-	+++
NAZW-12	Nf, Na	+++	3.4	+++	++
NAZW-13	C, Cm, Cx, Nf, Na	++	NT	+	+
NAZW-14	C, Cm, Nf	+++	3.8	+	
Total % of positive isolates		**100%**		**72.5%**	**100%**

Activity key: NH_3 & HCN production: High production (+++) , Moderate production (++), Low production (+), No production (-).

Seed Germination (%): +++ , 81-100% (significant over control) ; +/++, 61 – 80% (equal or slightly more over control). NT= not tested

Table- 6: Salt tolerance of isolated diazotrophs against salt and pH.

Tolerance traits	% of tolerant isolates		
	Group-A	Group-B	Group-C
Salt tolerance (% NaCl)			
0.5- 1.5	100	100	100
2.5	100	79	71
3.0	71	43	36
pH tolerance (pH of the medium)			
7.0-9.0	100	100	100
9.5	80	79	79
10	60	64	43

The growth behaviour of these diazotrophs was also evaluated against different salt concentrations (NaCl- 0.5-3.5%). The concentrations of 0.02% & 0.05% (present in Ashby's and Jensen's media respectively) were treated as controls. Among all 48 strains, no inhibition of growth was observed at 0.5-1.5% salt concentrations. At 2.5% salt concentration, growth was not inhibited in case of group-A strains. Whereas three strains (NAZ-7, NAZ-8 & NAZ-10) of group-B and four strains (NAZW-3, NAZW-10, NAZW-13 & NAZW-14) of group-C exhibited suppressed growth. At 3.0% salt concentrations, the percentage of tolerant strains was 71%, 43% and 36% of group-A, B & C respectively. However, all the strains were found to be less tolerant with increasing salt concentrations in the medium. At 3.5% NaCl only 25% of group-A and 14% each of group-B & C showed normal growth (Table - 6). Similar levels of salt tolerance among *Azotobacter* isolates were also reported by RajKumar[4].

Similarly, tolerance against pH (7.0-10.0) was also studied in the three groups of isolated strains. All the strains exhibited good growth at pH levels from 7.0 to 9.0. However, at pH 10.0, 43-64% of the strains were tolerant Our finding on pH tolerance were in agreement with the reports of Tippannavar *et al*.

Conclusion

On the basis of this preliminary investigation it may be concluded that Aligarh soil has a rich diversity of soil microorganisms. Asymbiotic diazotrophs show diversity and predominant forms may be grouped into three types viz., *Azotobacter chroococcum*, *Azotobacter* sp. I and *Azotobacter* sp. II. Some of these predominant forms exhibited promising plant growth promoting activities and relatively higher tolerance to pH & salt. Further characterization at genetic level and their relative efficiency in nitrogen fixation and plant growth promotion are needed to uncover their potential application as PGPR inoculant

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