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RESEARCH **P**APER

Deciphering soil diazotrophic diversity in the wheat-maize cropping system of Punjab using morphological, biochemical and molecular techniques

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Soil is the critical resource as well as a basic medium for the growth of natural micro flora present in it. The soil fertility depends on its physicochemical properties and microbial population diversity. Diazotrophs are the nitrogen fixing bacteria which possess *nif*H gene that is responsible for coding the nitrogenase enzyme involved in the reduction of atmospheric nitrogen to ammonia. These nitrogen fixing bacteria play an imperative role, function and significance in the soil. Soil samples were collected from the different wheat-maize cropping system of Punjab and analyzed for physicochemical properties as pH, electrical conductivity, organic carbon, soil texture, ammoniacal as well as nitrate nitrogen. Eighty diazotrophic bacteria were isolated on eight different nitrogen free media and characterized culturally, morphologically, biochemically, functionally and using molecular techniques. The diazotrophic nature of the isolates was confirmed by the amplification of *nif*H gene using two *nif*H primers *viz. nif*H1 and *nif*H2. The *nif*H positive isolates were further used for 16S rDNA restriction analysis using different enzymes such as *Taq*1, *Rsa* I and *Hae* III. Based on UPGMA clustering, the representative strains were sequenced and identified as *Bacillus amyloliquifaciens, Pseudomonas aeruginosa, Bacillus subtilis, Paenibacillus* sp., *Azotobacter vinelendii, Stenotrophomonas maltophilia, Rhizobium larrymorrei, Flavobacterium anhuiense, Sphingomonas paucimobilis, Paenibacillus panacisoli, Azospirillum* sp., *Pseudomonas putida, Paenibacillus amyloliticus, Bacillus circulans, Paenibacillus polymyxa* and *Xanthomonas oryzae.*

Key words : Diazotrophs, *nif* H, 16S rDNA sequencing, Physico-chemical properties, Soil

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INTRODUCTION

Soil is the basic medium which supports plant life by providing mechanical support and availability of nutrients. Assessment of soil quality generally consists of physical, chemical and biological components and their interaction with one another. Soil organisms are responsible for many processes that are essential for ecosystem function, including organic matter decomposition, nutrient cycling and particle aggregation (Wolters, 2001). Soil microorganisms influence above ground ecosystems by contributing to plant health, soil structure, soil fertility, soil formation, toxin removal and plant nutrition by the production of phytohormones and siderophores. The presence of plant growth promoting N_2-fixing bacteria and the possibility of a significant increase in plant performance and yield under nutrient limiting conditions by root-associated bacteria have been discussed for many years (Jha *et al.*, 2008). Biological nitrogen fixation is one of the most important environmental processes, which are carried out by a specific group of prokaryotes called diazotrophs. All organisms that can fix atmospheric N_2 belong to the group of diazotrophs.

The diazotrophs possess *nif H* gene which codes for the nitrogenase enzyme responsible for nitrogen fixation. Diversity of diazotrophs in the soil can be assessed by variety of methods which consist of conventional as well as molecular techniques. The conventional techniques include morphological characterization, utilization of carbohydrate

as sole carbon source (Garland,1996) and various biochemical tests to identify diazotrophs. The molecular approaches are successfully applied to analyze diazotrophic diversity in different soil systems to get information about the entire genetic material. The rhizosphere is the primary source for the microbial abundance, diversity and metabolic activity intimately related to the successful production of crops and maintenance of soil fertility (Vessey, 2003). The ability of diazotrophs to exhibit multiple plant growth promotional traits confers their candidature as potential plant growth promoting agents.

Keeping this in view, the present study was aimed to isolate and characterise diazotrophic bacteria from rhizospheric soils of wheat-maize based cropping system of Punjab.

RESEARCH METHODOLOGY

Soil physicochemical properties :

The soil samples were collected from the rhizosphere of different wheat-maize based cropping systems of Punjab. The soil samples were analyzed for various physicochemical properties such as soil texture, pH using potentiometric method (Jackson, 1973), electrical conductivity using Solubridge method (Richard,1954), organic carbon (OC) using rapid titration method (Walkley and Black, 1934) and mineral nitrogen content including ammonical and nitrate nitrogen using modified Kjeldahl method (Page *et al.*, 1982).

Isolation of diazotrophs :

Diazotrophic bacteria were isolated using serial dilution spread plate technique on eight different nitrogen free media *viz.*, Jensen's, Burks, Nitrogen free agar, Nitrogen deficient medium for *Klebsiella* and *Enterobacter*, *Derxia*, *Beijerinkia*, Dobereiner's medium and LGI medium. Pure colonies of bacterial cultures were isolated and sub cultured on their respective medium.

Cultural, morphological and biochemical characterization:

All the diazotrophic cultures were characterised culturally based on color, texture, margins and shape of the colony. Morphological characterization was done using Gram and metachromatic staining. The bacterial cultures were characterized biochemically by using standard techniques as described in *Bergey's manual of determinative bacteriology* such as oxidase, catalase, urease production, citrate utilization, methyl red (MR) and Voges-Proskauer (VP), starch solubilisation, indole production, nitrate reduction, triple sugar iron test (TSI) and H_2S production (Holt *et al.*, 1994).

Functional characterisation :

The plant growth promoting potential of various diazotrophic isolates was assessed on the basis of their ability to produce ammonia (Lata and Saxena, 2003), production of indole acetic acid (Gordon and Weber, 1951) and solubilisation of phosphate (Edi-Premono *et al.*, 1996).

Molecular characterisation

Isolation of DNA :

The isolation of genomic DNA from bacterial cultures was done by growing the cultures in SOC broth until late exponential phase. The pellet was obtained by centrifugation at 10,000 g for 10 minutes and to the pellet 567 µl TE buffer, 30 µl (10% w/v) SDS and 3 µl (20 mg/ml) proteinase K were added and the components were incubated for one hour at 37°C. Extraction buffer (100µl of 5M NaCl and 80µl of CTAB) was added to the solution and was incubated at 65°C for 10 min. An equal volume of chloroform/isoamyl alcohol was added and the upper aqueous layer was extracted with isopropanol (0.6 volume). The solution was incubated at -20°C for half hour, the pellet obtained was washed with 1 ml 70 per cent ethanol, air dried and dissolved in 100 µl of TE buffer.

***Nif* H and 16S rDNA amplification :**

The nitrogen fixing potential of diazotrophs was assessed using amplification of *nif* H genes using two *nif*H primer pairs: *nif*H1 (Forward: 5'-AAGGGCGGTATC GGCAAGTC-3' and Reverse: 5'-GCACGAAGTGGAT CAGCTG- 3') and *nif* H2 (Forward: 5'-TCTACGGAAA GGGCGGTATCGG-3' and Reverse: 5'GGCACGAAGT GGATC AGCTG-3'). PCR reaction mixture (15 µl) consisted of 1 X *Taq* polymerase buffer, 1.2 mM of $MgCl_2$, 0.2 mM of dNTPs, 20 pmol of each primer, 1 unit of *Taq* DNA polymerase and 100 ng of template DNA. The thermo cycling conditions for *nif* H consisted of an initial denaturation step at 95°C for 5 min followed by 28 amplification cycles of 95°C for 50 sec, 62°C for 1 min and 72^0C for 1 min and a final extension step of 72^0C for 10 min. The *nif* H positive isolates were subjected to amplification of 16S rDNA using universal 16S rDNA primer pair with the following sequence Forward: 5'-AGAGTTTGATCCTGGCTCAG-3' and Reverse: 5'-GGCTACCTTGTTACGACTT-3'. The PCR reaction mixture (50 µl) for 16S rDNA consisted of *Taq* polymerase buffer (1 X), $MgCl_2$ (1.2 mM), dNTPs (200µM), each primer (20pmol), *Taq* DNA polymerase (1U) and template DNA (100 ng). The thermo cycling conditions for 16S rDNA consisted of an initial denaturation step at 95^0C for 5 min 28 amplification cycles of 95^0C for 50 sec, 63^0C for 1 min and 72^0C for 1 min and a final extension step of 72^0C for 10 min. Restriction analysis of 16S rDNA was done using three restriction enzymes *Hae* III, *Rsa* I and *Taq* I. The restricted PCR products were run on 1.2% agarose gel. The scoring was done in a binary format and cluster analysis was performed using Unweighted Pair Group Method using Arithmetic averages (UPGMA) by

NTSYSpc 2.02 software (Rohalf, 1998). Based on various groups obtained in the dendrogram, the representative strains were sequenced using partial sequencing of 16S rDNA and related sequences obtained from GenBank database National Center for Biotechnology Information (NCBI) using BLAST, version 2 (Altschul *et al.*,1990) were aligned and the consensus sequence was computed using tblastx software. The phylogenetic tree was prepared using ClustalW.

RESEARCH FINDINGS AND ANALYSIS

The findings of the present study as well as relevant discussion have been presented under the following heads :

Soil physicochemical properties :

The texture of the soil samples collected from the different wheat-maize cropping system of Punjab varied from loamy sand to sandy loam with pH, 5.3-8.2 and EC, 0.18-0.79 ds/m. Punjab soils are generally deficient in organic carbon which ranged from 0.06-0.49 per cent in the soil samples. In this study, ammoniacal and nitrate nitrogen of the soil ranged from 49-130 mg/kg (Table 1).

Cultural and morphological characterisation :

On the basis of different cultural, morphological and physico-chemical characterisation, 80 diazotrophic isolates were selected for the further study. The cultural characteristics of different isolates varied from transparent to translucent white, creamy, yellow to lemon yellow and brown colored colonies. Majority of bacterial colonies were found to be mucoid and round with smooth margins whereas some of the colonies were non-mucoid type. Fifty-five isolates were found to be Gram negative whereas 25 were Gram positive. Majority of Gram positive isolates were found to be positive for metachromatic granule test (Table 2).

Bio-chemical characterisation :

A large number of isolates were positive for catalase

Table 1 : Physico-chemical properties of the soil samples collected from wheat-maize cropping system of Punjab

Sample no.	Cropping system	Texture	Physico-chemical properties				
			pH	EC (dSm^{-1})	OC (%)	NH_4+N (mg/kg)	NO_3-N (mg/kg)
1.	Wheat-maize	Loamy sand	7.7	0.44	0.13	70	130
2.	Wheat-maize	Loamy sand	7.6	0.79	0.30	49	91
3.	Wheat-maize	Sandy loam	7.9	0.44	0.13	72	120
4.	Wheat-maize	Loamy sand	7.7	0.46	0.18	80	120
5.	Wheat-maize	Loamy sand	5.3	0.18	0.17	110	90
6.	Wheat-maize	Loamy sand	8.2	0.30	0.06	50	70
7.	Wheat-maize	Loamy sand	7.1	0.59	0.31	70	90
8.	Wheat-maize	Loamy sand	7.8	0.51	0.49	28	84

Table 2 : Cultural and morphological characteristics of diazotrophic bacteria isolated from wheat-maize cropping system of Punjab

Isolates	Cultural	Shape	Gram	Metachromatic
WM-01	Transparent, mucoid, round, entire	Rods	+	+
WM-02	Transparent, mucoid, round, entire	Rods	+	+
WM-03	Transparent, mucoid, elevated, round	Rods	-	+
WM-04	Creamy round and non-pigmented	Cocci	-	+
WM-05	Whitish, elevated and intricate colonies	Rods	-	+
WM-06	Transparent, mucoid, smooth margins	Rods	-	+
WM-07	White, mucoid, round, furrowed margins	Rods	-	-
WM-08	Yellow, non-mucoid, round, entire	Rods	+	-
WM-09	Thick, creamy, mucoid, large, raised	Rods	-	+
WM-10	White, mucoid, oval, large, flat, rods	Rods	-	+
WM-11	White, non-mucoid, entire, small	Spirillum	-	+
WM-12	Transparent, smooth, small dot like	Rods	-	+
WM-13	Transparent, mucoid, round, entire	Rods	-	+
WM-14	Peach, mucoid, polymorphic, entire	Coccobacilli	+	+
WM-15	Round, elevated, mucoid, rods	Rods	+	+
WM-16	Round, elevated, spreading entire	Cocci	-	+

+, Growth, --, no growth

and oxidase test except one isolate (WM-04) which was catalase negative and two isolates (WM-13 and WM-15) were positive for oxidase test. All the isolates were found to be positive for methyl red and urease test except WM-04, WM-05, WM-06, WM-11, WM-15 and WM-16 that were negative for methyl red test. The isolates were found to be negative for Voges Proskauer and H_2S production except one isolate (WM-15) that was found to be positive for Voges Proskauer test. Six isolates (WM-07, WM-08, WM-09, WM-11, WM-12 and WM-14) were showed positive results for indole production and seven isolates (WM-07, WM-08, WM-09, WM-11, WM-12, WM-14 and WM-15) were showing positive results for citrate utilization, However, the isolate WM-11 was weakly positive for the same. Majority of the isolates were found to be negative for starch solubilisation while five isolates (WM-02, WM-05, WM-08, WM-13 and WM-14) were positive for

Table 3 : Biochemical characteristics of diazotrophic bacteria isolated from wheat-maize cropping system of Punjab

Isolates	Cat	Oxi	MR	VP	Ind	Cit	Ure	Sta	Nit	TSA
WM-01	+	+	+	-	-	-	+	-	+	Glu. Ferm.
WM-02	+	+	+	-	-	-	+	+	-	Glu, Suc, lac ferm
WM-03	+	+	+	-	-	-	+	-	+	Glu, Suc, lac ferm
WM-04	-	+	-	-	-	-	+	-	+	Glu. Ferm.
WM-05	+	+	-	-	-	-	+	+	+	Glu, Suc, lac ferm
WM-06	+	+	-	-	-	-	+	-	+	Glu, Suc, lac ferm
WM-07	+	+	+	-	+	+	+	-	+	No change
WM-08	+	+	+	-	+	+	+	+	-	No change
WM-09	+	+	+	-	+	+	+	-	+	Glu, Suc, lac ferm
WM-10	+	+	+	-	-	-	+	-	-	No change
WM-11	+	+	-	-	+	+	+	-	+	Glu, Suc, lac ferm
WM-12	+	+	+	-	+	+	+	-	+	Glu. Ferm.
WM-13	+	-	+	-	-	-	+	+	-	Glu, Suc, lac ferm
WM-14	+	+	+	-	+	+	+	+	-	Glu, Suc, lac ferm
WM-15	+	-	-	+	-	+	+	-	-	Glu. Ferm.
WM-16	+	+	-	-	-	-	+	-	+	No change

+, Growth, --, no growth, W, Weak, Glu, glucose, Suc, sucrose, Lac, lactose, Ferm, fermentation
Cat- Catalase, Oxi- Oxidase, MR- Methyl red,VP- Voges Proskauer, Ind- Indole Production, Cit- Citrate Utilization, Ure- Urease, Sta- Starch Utilization, Nit- Nitrate reduction, TSA- Triple sugar iron agar assay

Table 4 : Functional characteristics of different diazotrophic isolates

Isolate No.	Phosphate solubilisatiion (mm dia zone)	IAA production (µg/ml)	Ammonia production
WM-01	09	33.0	+
WM-02	08	26.0	+
WM-03	11	23.0	+
WM-04	09	11.2	+
WM-05	09	15.0	+
WM-06	-	20.0	+
WM-07	08	23.0	+
WM-08	-	25.0	+
WM-09	-	12.0	+
WM-10	10	16.0	+
WM-11	-	35.7	+
WM-12	-	18.0	+
WM-13	09	22.0	+
WM-14	-	27.0	+
WM-15	09	15.6	+
WM-16	08	12.8	+

+, Growth; -, no growth

this test. Ten isolates (WM-01, WM-03, WM-04, WM-05, WM-06, WM-07, WM-09, WM-11, WM-12 and WM-16) were identified as positive for nitrate reduction test. Twelve isolates (WM-01, WM-02, WM-03, WM-04, WM-05, WM-06, WM-09, WM-11, WM-12, WM-13, WM-14 and WM-15) exhibited variable results for TSA medium test, thereby indicating their ability to ferment different sugers *i.e.* sucrose, lactose and glucose (Table 3).

Molecular characterisation of diazotrophs :

All the isolates were screened for their ability to amplify *nif* H gene using two *nif* H primers as *nif* H1 and *nif* H2. The amplification resulted in the formation of a *nif* H product at 610 bp. Thirty isolates were found to show amplification of *nif*H gene with two *nif* H primers. On the basis of *nif* H screening, 30 isolates were subjected to 16S rDNA analysis. A successful amplification of ~1500 bp fragment of 16S rDNA was obtained from all the isolates. The restriction analysis of amplified 16S rDNA resulted in the formation of different fingerprinting profiles using three restriction enzymes *Taq* I, *Rsa* I and *Hae* III (Fig. 1). The UPGMA clustered the 41 isolates into four major groups and one separate lineage (Fig. 2). Cluster I was subgrouped into three groups *viz.*, Ia, Ib and Ic. Group Ia consisted of 13 isolates: WM-07, WM-13, WM-20, WM-36, WM-24, WM-09, WM-29, WM-31, WM-15, WM-26, WM-32, WM-11 and WM-03. An isolate WM-03 was quite different from other isolates of group Ia and joining to other isolates at 55 per cent similarity co-efficient. Group Ib consisted of eight isolates (WM-17, WM-39, WM-18, WM-22, WM-01, WM-30, WM-19 and WM-25) which were grouped at 55 per cent similarity co-efficient. In a similar way, cluster Ic consisted of four isolates (WM-05, WM-33, WM-28 and WM-08) grouped at 52 per cent, similarity co-efficient. Further subdivisions of three groups showed a narrow base among the isolates within a group. Cluster II and III consisted of five (WM-23, WM-34, WM-02, WM-38 and WM-14) as well as seven (WM-04, WM-40, WM-27, WM-10, WM-35, WM-14 and WM-41) isolates which were grouped at 50 per cent and 46 per cent similarity co-efficient. Similarly, three isolates (WM-37, WM-12 and WM-16) were present in group IV, which were grouped at 43 per cent similarity. An isolate WM-06 was showing a separate lineage. All the isolates were joining together at 35 per cent similarity co-efficient. The diversity analysis showed that isolates collected from different region of Punjab from wheat-maize cropping system differed from each other genetically. The phylogenetic tree of representative cultures is presented in Fig. 3. The identified cultures were found to be closely related to species of genus *Bacillus amyloliquifaciens* (WM-02), *Pseudomonas aeruginosa* (WM-07), *Bacillus subtilis* (WM-01), *Paenibacillus* sp. (WM-08), *Azotobacter vinelendii* (WM-10), *Stenotrophomonas maltophilia* (WM-03),

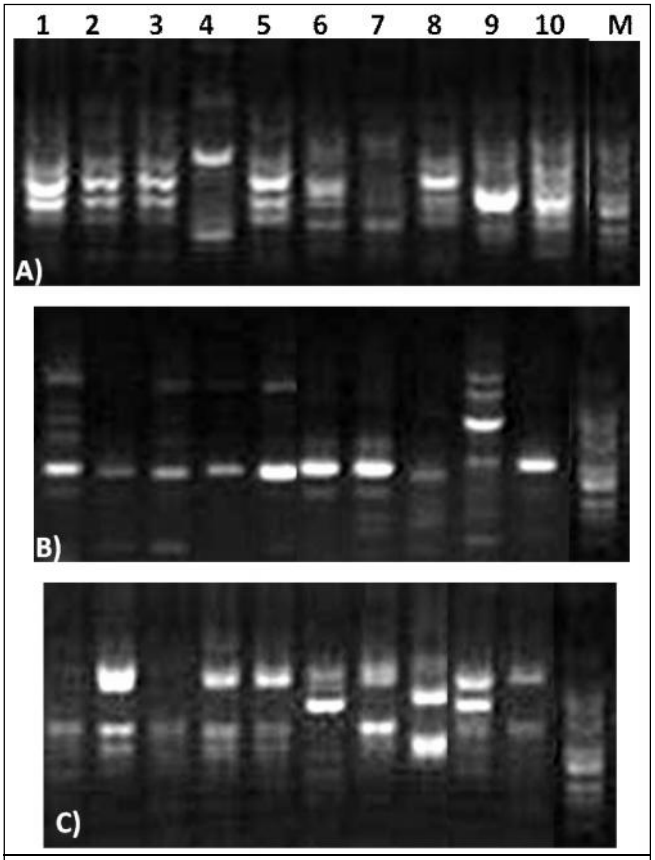

Fig. 1 : Restriction patterns of PCR amplified fragments of 16s rDNA digested with A) *Hae*III B) *Taq*I and C) *Rsa*I

Rhizobium larrymorrei (WM-04), *Flavobacterium anhuiense* (WM-05), *Sphingomonas paucimobilis* (WM-06), *Paenibacillus panacisoli* (WM-09), *Azospirillum* sp. (WM-11), *Psudomonas putida* (WM-12), *Paenibacillus amyloliticus* (WM-13), *Bacillus circulans* (WM-14), *Paenibacillus polymyxa* (WM-15) and *Xanthomonas oryzae* (WM-16).

Functional characterisation :

The different isolates were evaluated for functional chacterisation using phosphate solubilisation, IAA production and ammonia production. From the identified isolates, ten isolates (WM-01, WM-08, WM-03, WM-04, WM-05, WM07, WM-10, WM-13, WM-15 and WM-16) showed the development of sharp phosphate solubilisation zones on Pikovskaya's medium. The production of IAA by diazotrophic isolates varied from 11.2-35.7 µg/ml. The maximum IAA production (35.7 µg/ml) showed by isolate WM-11. All isolates produced ammonia in peptone water using Nessele's reagent and colour change in broth, from yellow to brown, which was indicative of ammonia production (Table 4).

The texture of the soil samples depend upon the particle size that dominates its makeup. Dhaliwal (1991) also reported

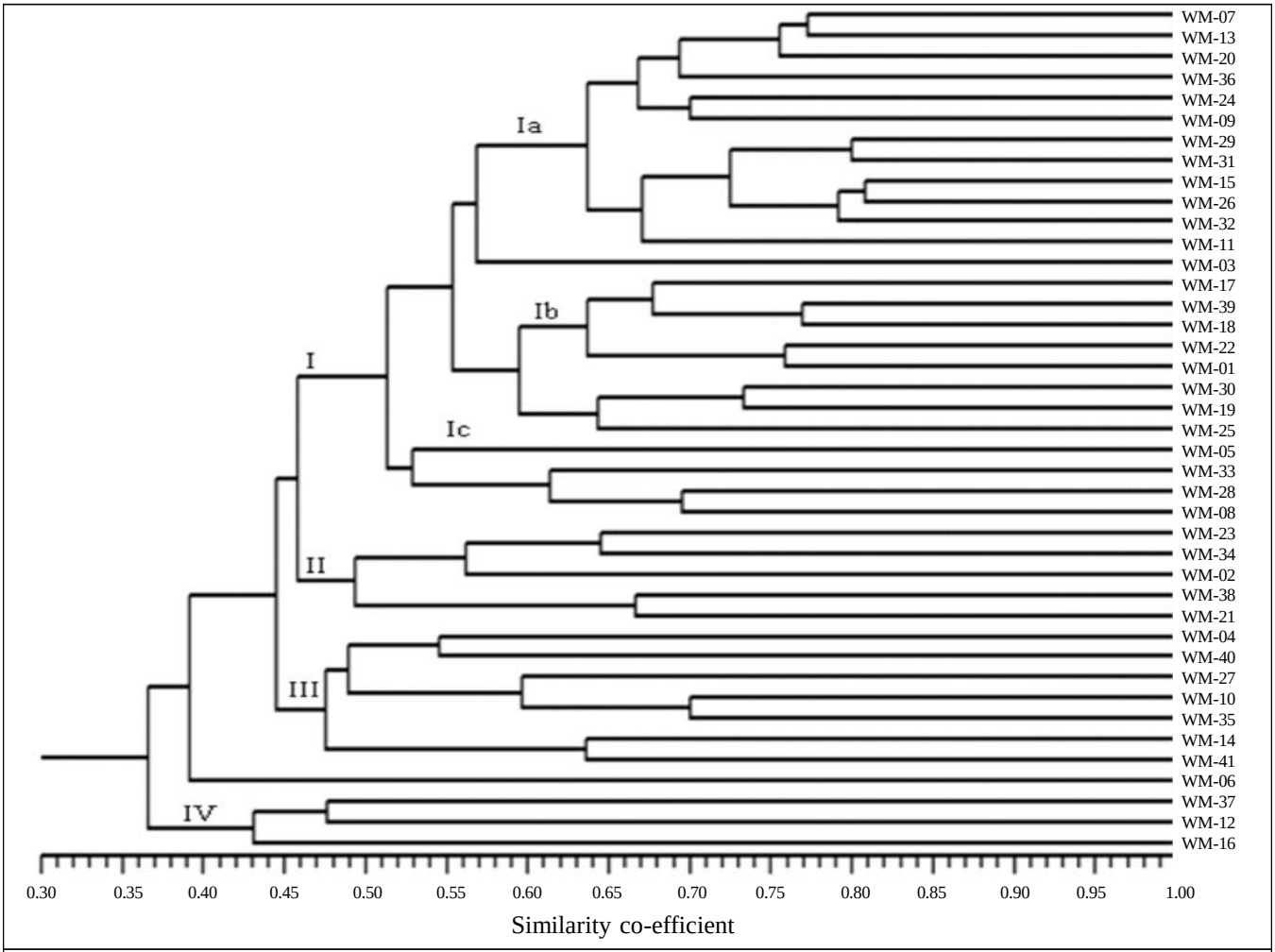

Fig. 2 : Dendrogram based on UPGMA clustering showing 41 isolates

the texture class from the flood plain region of Punjab. All the fertile soils have at least small amount of soluble salts. Carbon is the main constituent of soil organic matter and the organic matter is directly based upon the organic carbon. The results were supported by the observations of (Anand, 1974), who concluded that organic carbon content in the soils of Punjab varied from 0.02-0.95 per cent. The low amount of ammoniacal and nitrate nitrogen favors the growth and count of bacteria (Somani, 2005) due to which the microbial count was low in the soils with high ammoniacal and nitrate nitrogen. The diversification of diazotrophic bacteria was done on eight different nitrogen free media (Jha *et al.*, 2008 and Islam *et al.*, 2010). Majority of isolates exhibited mucoid texture due to the production of exopolysacchrides, which helps in the protection of bacteria against dessication, phagocytosis and phage attack besides supporting nitrogen fixation by preventing high oxygen tension (Tank and Saraf, 2003). The presence of diazotrophs in the rhizosphere of wheat maize cropping system is well known due to their ability to

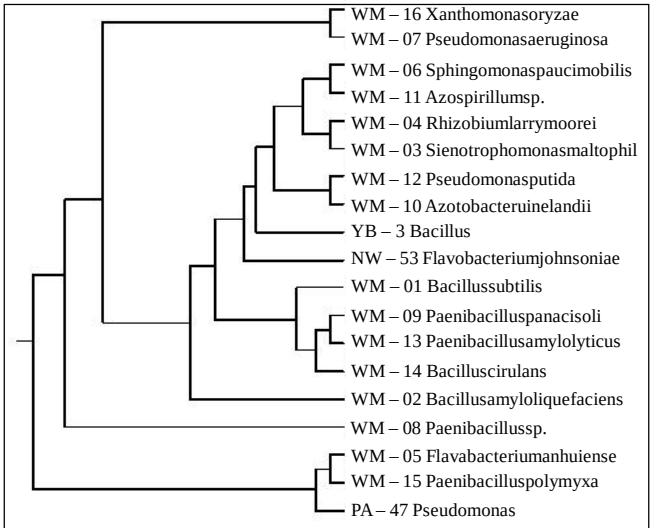

Fig. 3 : Phylogenetic tree of identified diazotrophic cultures

exhibit various plant growth promotional traits which enhance plant growth, development and maintain health. The screening for diazotrophy among various isolates was done using *nif* H, since it is highly conserved in nature and codes for an essential subunit of a widely distributed nitrogenase enzyme (Burris and Roberts, 1993). All the isolates were able to grow and subculture on nitrogen free media, however, only few gave amplification with *nif*H primers, which could be mainly due to defects in the expression of *nif* H and requirement of rhizobacterial assistance in the vicinity for the expression of *nif* H genes (Ozawa *et al*., 2003). The above results are supported by the observations of Kuklinshy-Sobral *et al.* (2004); Chowdhury *et al.* (2007) and Islam *et al.* (2010) where *nif* H amplification was observed in only few isolates. Characterization of isolates based on cultural, morphological and biochemical characteristics revealed the presence of genera *Bacillus*, *Pseudomonas*, *Rhizobium* and *Azotobacter*. The identification of various bacteria using biochemical characterization has been done for *Azotobacter*, *Pseudomonas* (Selvakumar *et al.*, 2009), *Bacillus* and *Rhizobium*. Molecular techniques have advantage of estimating the diversity of nitrogen fixing bacteria with greater precision. Molecular characterization based on amplification of 16S rDNA revealed the presence of *Paenibacillus polymyxa, Klebsiella pneumoniae, Burkholderia cenocepacia, Rhizobium* sp., *Bacillus megaterium, Pseudomonas* sp., *Agrobacterium tumefaciens, Stenotrophomonas maltophilia, Bacillus subtilis* and *Paenibacillus* sp. The taxonomic affiliation of diazotrophic bacteria from wheat based cropping system revealed the dominance of alpha proteobacteria, beta proteobacteria and firmicutes. Similar results were also obtained by (Chowdhury *et al.*, 2007).

Conclusion :

The bacterial isolates were characterised using cultural, morphological and biochemical characteristics as diverse genera of *Azotobacter*, *Pseudomonas*, and *Bacillus*. Based on partial sequencing of 16S rDNA representative cultures from were identified as *Bacillus amyloliquifaciens, Pseudomonas aeruginosa, Bacillus subtilis, Paenibacillus* sp., *Azotobacter vinelendii, Stenotrophomonas maltophilia, Rhizobium larrymorrei, Flavobacterium anhuiense, Sphingomonas aeroginosa, Paenibacillus panacisoli, Azospirillum* sp., *Pseudomonas putida, Paenibacillus amyloliticus, Bacillus circulans, Paenibacillus polymyxa* and *Xanthomonas oryzae.* Functional characterization revealed the presence of ammonia producers, IAA producers, and P solubilizers.

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