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Bacterial Flora of Arachis Hypogea Plants from Agricultural Fields of Punjab, Pakistan

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Abstract

The study was carried out to identify the rhizo-microorganisms from the roots of Arachis hypogea plants collected from agricultural fields of upper Punjab (Chakwal), Pakistan. Eight purified bacterial isolates were further identified by 16S rRNA gene sequencing. Bacillus spp were found to be most abundant in the rhizophere of Arachis hypogea. The isolated bacteria were mostly mesophilic (grow at 37°C and 42°C) and resistant to chloramphenicol. All isolates were found to be resistant to metals such as Mn, Pb, Co and Cr (100 µg/ml). However, these were sensitive to Hg and Cd at all the concentrations studied (100, 500 & 1000 µg/ml). Almost all isolates were sensitive to all metals at higher concentrations except few isolates which were resistant to Zn. The plant growth promoting ability was tested for Arachis hypogea seeds in pot experiment. No nodule formation was observed with any of the isolates while all of them promoted the growth of Arachis hypogea.

Keywords: Rhizosphere; Bacteria; *Arachis hypogeal;* Metal Resistance; Antibiotics; Acid Phosphatase.

1. Introduction

The soil ecology is complex and regulated by the numerous physical, chemical and biological factors [1]. The microbial communities of the rhizosphere contribute a wide range of essential services such as decomposition, mineralization and nutrient availability, influence the fertilizers reserves and soil toxicity[2]. The nutrient cycles are helpful for the healthy growth of plants[3]. Rhizospheric interaction are one which based on dead plant material which affects nutrient flows and energy and the other based on living plants and roots.

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The rhizospheric microbes are influenced by root exudation, plant growth stages and crop rotation [4]. Certain microorganisms colonize in root tissues which derives their (energy source) carbon from decaying plant material under the influence of root exudates. Microbial activity in the rhizosphere affects rooting patterns and supply nutrients and root exudates[5]. Peanut (*Arachis hypogea*) is an annual herbaceous plant which thrives in tropical and sub-tropical climates it is one of the oil seed crop of rain fed areas which is rich in number of phytonutrients. Its rhizospheric bacterial community has a potential to provide useful bio-inoculants for ecofriendly sustainable green agriculture practices[6]. Thus there is a dire need to understand the microbial structure and activity in rhizosphere of *Arachis hypogea*. The present study was planned to explore the biodiversity of rhizospheric bacteria of agricultural fields in upper Punjab which is the arid zone, where *Arachis hypogea* is cultivated as a common practice. The objective of the study was to explore the presence of plant growth promoting rhizobacteria and to study its effect on the growth of *Arachis hypogea*

2. Materials & Methods

2.1 Sample Collection

Eight peanut plants along-with their roots, nodules and adhering rhizospheric soil were plucked from the agricultural fields of upper Punjab (Chakwal, Pakistan). These plants were shifted to plastic bags in sterilized condition, labeled and transported to the laboratory. The soil adhering to the roots was carefully collected in a separate container and processed.

2.2 Isolation of Bacteria from Different Sources

Serial dilutions of soil were prepared using 1% soil suspension and cultured on L-agar plates at 37°C for 24 hours in an aerobic environment. Growth thus obtained was further purified by restreaking. Rhizoplane from roots of *Arachis hypogea* plants were cultured on L- agar plates by touch plate methods. The roots and nodules of plant *Arachis hypogea* were also cultured. The washed plant roots and nodules were chopped and sterilized using 0.1% $HgCl_2$ solution. This material was crushed and their extract was cultured. The purified bacteria were identified by colony morphology. The bacterial cell morphology was studied regarding size, shape, arrangement and Gram staining reaction. In addition to it motility, capsular staining on young cultures and spore staining was performed on a week older cultures incubated at 37°C. The identification of bacteria was based on colonial, morphological, biochemical, physiological and molecular characteristics as described by Gerhadt and his colleagues [7]. The purified cultures were also studied on differential medium such as MacConkey agar and Eosin Methylene blue agar. The biochemical tests performed include catalase test, starch hydrolysis, citrate utilization test, Methyl red test, Voges Proskauer test, H_2S production test and oxidation fermentation test. Physiological characteristics such as effect of pH (pH 3-9) and effect of temperature (20°C, 37°C and 42°C) on bacterial growth were also studied. The metal tolerance profile of bacterial isolates were recorded by culturing on L-agar plates supplemented with 100, 500 and 1000 µg/ml conc. of Mn^{+2} ($MnSO_4$), Ni^{+2} ($NiSO_4$), Zn^{+2} ($ZnSO_4$), Pb^{+2} [$Pb(NO_3)^2$], Cr^{+6}, Cu^{+2} ($CuSO_4$), Co^{+2} ($CoCl_2$), Hg^{+2} ($HgCl_2$), Ag^{+1} ($AgNO_3$), Cd^{+2} ($CdCl_2$). Antibiotic resistance of the bacterial isolates was studied by the addition of antibiotics in media. The antibiotic tolerance for ampicillin at concentration of 300 µg/ml, tetracycline 25µg/ml, streptomycin 500 µg/ml and chloramphenicol 5 µg/ml were studied.

2.3 16S rRNA Sequencing

Molecular identification of some of the isolated isolates designated NA, NB, NG, NI, CrA, CrC, CrF and CrG were done by sequencing of 16S rRNA percent homology using MEGABLAST at NCBI.

2.4 Effect of Bacterial Isolates on Germination and Growth of Plants

The impact of bacterial inoculation on the germination and growth parameters of *Arachis hypogea* were studied in laboratory at room temperature. Sterile seeds of *Arachis hypogea* were germinated on filter paper impregnated with bacterial suspensions. All plates were kept in dark for three days and then placed in light. Germination process was noticed daily. Seedlings were grown in pots for 30 days. The experiments was conducted in three replicates. Seedlings were removed and growth parameters were recorded such as seed germination, fresh weight and dry weight etc. In plant material acid phosphatase enzyme was measured following the method of Iqbal and Rafique (1987).

3. Results

In total 8 isolates of bacteria were identified as per identification scheme of Bergey's Manual (Table 1). Briefly the color of the colonies was white to off-white, round with irregular margins and sizes varied from pinpoint to 2.5 cm. The colonies isolated from roots (CrA and CrC) were round while two other colonies (CrF and CrG) were irregular in shape. The colonies purified from crushed nodules were round in shape except NB which was lobulated in shape. Majority of the colonies had flat elevation. All the isolates have opaque colonies except CrC, which has transparent colonies. The texture of half of the colonies was smooth and other half had rough texture. Colonial characteristics of different isolates are presented in Table 2. As all the isolates isolated were gram positive bacilli and were spore former except CrA. Most of isolates were capsulated except isolates CrA, and NG as shown in Table 2. All the isolated isolates were successfully grown over EMB agar. On the other hand only CrF could be grown on MacConkey agar while rest of the isolates failed to do so. Only two isolates i.e. CrC and NB had the ability to utilize citrate. All the isolates were catalase positive. The strain NI had the ability to produce H_2S. All the isolates had the ability to hydrolyze starch except CrA, CrG, NG and NI. Variable results for IMVIC tests were recorded. The isolates which were urease negative included CrC, and CrF while other isolates were positive for urease enzyme. The sugar fermentation reaction indicated that all the isolated isolates were facultative anaerobes as shown in Table 4 (A) and 2.

3.1 Heavy Metal and Antibiotic Resistance

All the isolated isolates were resistant to heavy metals such as Mn, Pb, Co and Cr up to100 µg/ml (Table 4). However, majority were sensitive to different metals at 500 µg/ml or more concentration except few isolates which were resistant to zinc even at 1000 µg/ml and all were sensitive to Hg and Cd at all concentrations. For antibiotic resistance it was observed that all the isolates were resistant to chloramphenicol (5µg/ml) while twenty of these were resistant to ampicillin (300 µg/ml) five were resistant to streptomycin (500 µg/ml) and four were resistant to tetracycline (25 µg/ml) as shown in table 3.

Table 1: Identification and characterization of *Arachis hypogea* associated bacterial isolates

Isolates	Location	Closest Matched Species	% homology	Source
NA	Chakwal	*Bacillus cereus*	100%	Root rhizoplane
NB	Chakwal	*Bacillus subtilus*	99%	Root rhizoplane
NG	Chakwal	*Bacillus megaterium*	99%	Root rhizoplane
NI	Chakwal	*Bacillus simplex*	99%	Root rhizoplane
CrA	Chakwal	*Bacillus cereus*	99%	Root rhizoplane
CrC	Chakwal	*Bacillus cereus*	96%	Root rhizoplane
CrF	Chakwal	*Bacillus cereus*	98%	Root rhizoplane
CrG	Chakwal	*Bacillus* sp.	99%	Root rhizoplane

Table 2: Morphological and biochemical characterization of *Arachis hypogea* associated bacterial isolates

Characteristics	ISOLATES							
	CrA	CrC	CrF	CrG	NA	NB	NG	NI
Cellular morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Gram's Staining	+	+	+	+	+	+	+	+
Capsular staining	-	+	+	+	+	+	+	+
Spore staining	-	+	+	+	+	+	_	+
Growth on EMB agar	+	+	+	+	+	+	+	+
Growth on MacConkey	-	-	+	-	-	-	-	-
Growth on citrate agar	-	+	-	-	-	+	-	-
Catalase	++	++	+	++	+	+	+++	+
Oxidase	+	+	+	-	+	+	-	+
Motility	-	+	+	-	+	+	+	-
H_2S production	-	-	-	-	-	-	-	+
Starch hydrolysis	-	+	+	-	+	+	-	-
Methyl red	-	+	+	-	+	-	-	-
Vogues Prausker	+	+	+	-	+	-	-	+
Indole	-	-	-	-	-	-	-	-

Table 3: Antibiotic resistance profile of bacterial isolates

Characteristics	ISOLATES							
	CrA	CrC	CrF	CrG	NA	NB	NG	NI
Ampicillin (300 µg ml^{-1})	-	+	+	-	+	+	+	+
Chloroamphenicol (5 µg ml^{-1})	+	+	+	+	+	+	+	+
Streptomycin (500 µg ml^{-1})	-	-	-	-	-	-	-	+
Tetracycline (25 µg ml^{-1})	-	-	-	-	-	-	-	-

Table 4: Heavy metal resistance profile of bacterial isolates

Characteristics	ISOLATES							
	CrA	CrC	CrF	CrG	NA	NB	NG	NI
Cr (100 µg ml^{-1})	500	100	100	100	500	100	500	100
Mn (100 µg ml^{-1})	100	100	100	100	100	100	100	100
Cd (100 µg ml^{-1})	100	100	100	-	100	100	-	-
Pb (100 µg ml^{-1})	100	100	100	100	100	100	100	100
Co (100 µg ml^{-1})	100	500	500	100	500	100	100	100
Hg (100 µg ml^{-1})	100	-	-	-	-	-	-	-
Zn (100 µg ml^{-1})	1000	1000	1000	100	100	-	100	100

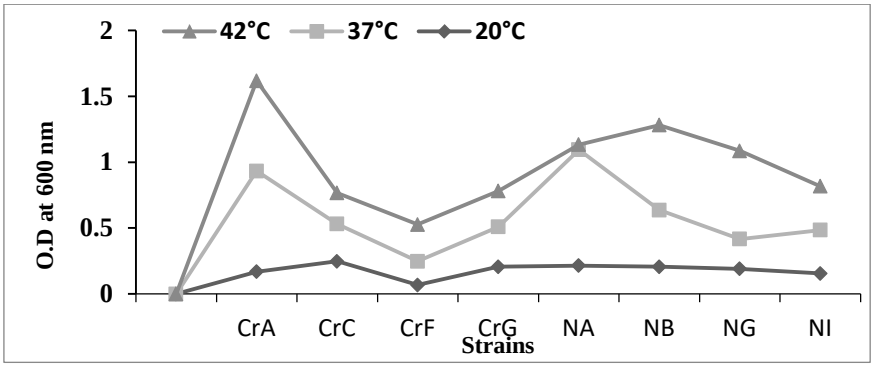
Table 5: Impact of bacterial isolates on the various growth parameters of *Arachis hypogea* plants.

Isolates	Plant Growth parameters			
	% germination	Fresh weight (g)	Dry weight (g)	Acid phosphatase content (unit g^{-1} fresh weight)
Control	90	212.6±9.5	8.83±0.56	70.3±2.3
CrA	90	232.5±7.9	8.98±0.34	65.6±2.9
CrC	95	213.8±11.5	7.87±0.25	78.8±3.7
CrF	100	227.6±15.8	8.96±0.45	62.3±2.5
CrG	100	278.4±14.3	9.20±0.32	71.2±4.0
NA	100	242.9±16.9	9.45±0.51	59.5±3.9
NB	95	214.4±13.0	8.53±0.34	67.2±4.1
NG	95	264.0±12.6	9.56±0.41	76.3±3.4
NI	100	233.7±11.8	8.67±0.38	67.0±3.0

3.2 Physiological Characteristics

Bacterial growth was recorded on all the three temperatures i.e 20°C, 37°C and 42°C. Optimum growth temperature of isolates varied. Isolates were thus classified as mesophiles as maximum growth was observed at 37°C to 42°C (Fig 1A). The effect of pH was studied by culturing at pH of 3, 5, 7 and 9. Although all of the isolates showed some growth at all the pH range but maximum growth was observed at neutral pH (pH 7) thus classified neutrophiles (Fig 1B).

A.



B.

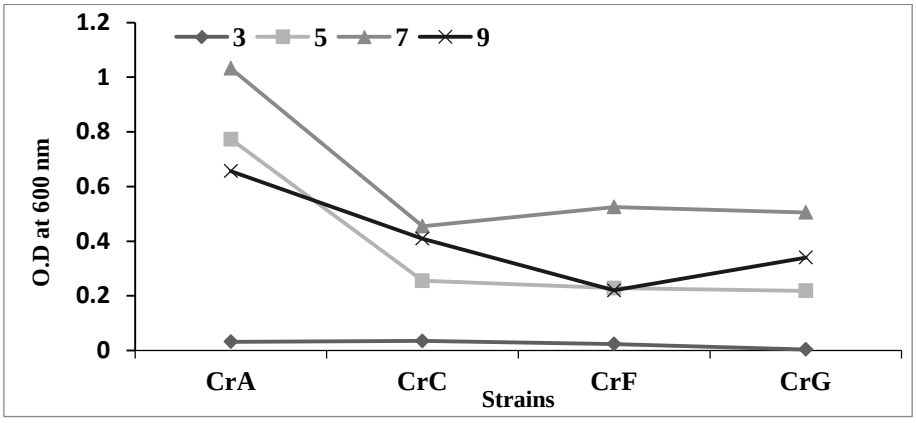


Figure 1: Growth of bacterial isolates at various temperatures-A (20, 37 and 42^{o}C) and pHs-B (3, 5, 7 and 9) after 24 hours of incubation periods.

3.3 Genetic Characterization

These isolates were subjected to ribotyping using 16S rRNA gene sequencing and isolates NA, NB, NG, NI, CrA, CrC, CrF and CrG had close homology with genus *Bacillus*. The isolates NA, CrA, CrC, CrF had highest

homology with *Bacillus cereus*, strain CrG with *Bacillus* species and other isolates were homologous to *Bacillus megaterium* (NG), *Bacillus subtilus* (NB) and *Bacillus simplex* (NI) (table 1).

3.4 Plant Microbe Interaction Experiments

The impact of these isolates was also studied on the growth of *Arachis hypogea* plants in pot experiments. The results showed that majority of bacterial inoculation resulted an increase in seed germination and fresh weight of plants as compared to control (Table 5). Few isolates (CrC, CrG) caused an increase while others have inhibited the acid phosphatase content as compared to control plants.

4. Discussion

In the present study the rhizosphere and rhizoplane of plant *Arachis hypogea* collected from the arid agricultural fields of Pakistan were cultured to identify the diverse bacterial community well adapted to the local environment. The selected microbes from such populations could further be explored for their plant growth promoting potential. Identification and understanding the effect of indigenous beneficial microbial communities could pave the way to modify the agricultural practices such as crop rotation and management of agricultural fields to enhance soil fertility and increased productivity. In the present study the purified organisms were also studied for their potential to enhance the germination and growth on one of the important crop *Arachis hypogea* of this region. Approximately 28 diverse aerobic microbes were initially isolated in the present study. However, eight had close homology with genus *Bacillus* including the species of *Bacillus cereus* (NA, CrA, CrC, CrF), *Bacillus megaterium* (NG), *Bacillus subtilus* (NB), *Bacillus simplex* (NI) and other *Bacillus* species. Bacilli are reported to ubiquitous soil bacteria particularly *Bacillus megaterium* and *Bacillus cereus* [8].

The microorganisms living in close to the roots may have a direct effect on plant growth by providing plants with certain nutrients such as increased solublization of inorganic phosphate, iron nutrition, production of phytohormones and certain other volatile compounds. The indirect effect of isolates includes antibiotics activities against pathogens and also compete with other growth suppressing microbes in the soil [2]. The rhizobacteria alters the solubility and availability of mineral nutrients and thus contribute in the plant health. The round and white to off-white colonies observed are the growth characteristic of *Bacillus* spp. The colonies purified from crushed roots CrA, and CrC, were round in shape while colonies of CrF and CrG were irregular in shape. The colonies obtained from the crushed nodules were all round except NB which was lobulated.

The round colonies are reported to be the characteristic of most of the *Bacillus* spp. while other rhizobacteria form irregular colonies. Two of the common spore former genera are Bacillus and Clostridium while we were unable to grow Clostridium which was not unexpected because our cultures were aerobic while other workers also reported these organisms as well. Growth of bacteria was also carried on differential media EMB and MacConkey. All the isolates showed growth on EMB agar. Among the isolates isolated only two isolates CrC and NB were positive for citrate utilization test in contrast with previous reports where citrate utilization of bacillus species and other rhizobacteria were reported [9].

The isolates CrA, CrG, NG and NI were devoid of the ability to hydrolyze starch while rest did so. Starch

hydrolysis is reported to be the characteristics of rhizospheric bacteria. Rhizospheric bacteria used both monosaccharides and polysaccharides in order to maintain their nutrient requirements [10]. Thus on the basis of these biochemical and morphological tests isolates NA, NB, NG, NI, CrA, CrC, CrF, CrG were closer to the family Bacillaceae. *Bacillus* is being reported to be the predominant diazotrophic genera in the rhizosphere in number of plants [10]; while *Pseudomonas* predominates in the rhizosphere of non diazotrophic species [11]. Endophytic microflora has complex relationship with the plants and may produce growth factor which competes with other microbes [12]. The presence of aerobic spore forming bacteria are generally recognize as free living soil organisms belonging to the family Bacillaceae [13]. Their antagonistic activity protects the plants from phytopathogenic microbes [14].

Many endophytic bacilli including *B. cereus*, *B. pumilus* and *B. subtilus* produce hydrolytic enzymes and antibiotics. Acid phosphate was estimated in the plant material inoculated with the isolates NA, NB, NG, NI, CrA, CrC, CrF, and CrG separately. Different amount of acid phosphatase was present in each plant inoculated with different isolates separately. Majority of isolates showed less amount of acid phosphatase as compared to control plant while few other isolates showed some increment in amount of acid phosphatase as compared to control plant. The difference in the amount of acid phosphatase indicated that different isolates have different effect on the plant growth that's why the amount of phosphatase was different in the plant material inoculated with different isolates.

5. Conclusion and Recommendation

We can conclude from above study that valuable growth in plants is observed while studying the effects of bacteria. The bacteria are specially isolated from soil, promotes plant growth. The microorganisms living in close to the roots may have a direct effect on plant growth by providing plants with certain nutrients such as increased solublization of inorganic phosphate, iron nutrition, production of phytohormones and certain other volatile compounds. Majority of growth promoting bacteria shows affiliation with Bacillus.

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